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Prematurity and Early Life Programming

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Contents

Abstract

Preterm infants are at increased risk of cardiometabolic and neurodevelopmental disorders in later life. The typical postnatal growth pattern of failure to achieve the equivalent of a normal fetal growth rate, followed up by catch-up growth, altered adiposity and altered hypothalamic-pituitary-adrenal axis (HPA) activity may be predisposing factors. Potential mechanisms that may mediate such programmed effects include altered DNA methylation and faster telomere attrition.

A prospective cohort of 46 very preterm (25+2 to 31+5 weeks' gestation, mean 28.6) and 40 full term (38+3 to 42+2 weeks' gestation, mean 40.2) infants was established to investigate potential mechanisms. Infants were studied at birth, term equivalent age, 3 months and 1 year corrected for prematurity. At all time points, linear growth and body composition (by densitometry) were measured and buccal (epithelial) cells was collected for measurement of DNA methylation (5mC) and relative telomere length.

Compared with full term infants, preterm infants were lighter ($p < 0.001$) and had a smaller head circumference ($p < 0.05$) at all time-points and were shorter at term equivalent ($p < 0.001$) and 3 months corrected age ($p = 0.002$). Preterm infants also had greater percentage body fat at term equivalent age (mean difference = 5.5%, $p < 0.001$), which normalised by 3 months corrected (mean difference = 0.9%, $p = 0.4$). Preterm infants had a blunted salivary cortisol response (mean difference 0.4 μg/dL, $p = 0.02$) to a stressor (physical examination) at 3 months compared to term infants at this age, suggesting altered activity of the HPA axis.

5mC is fundamental in the control of expression of imprinted genes involved in fetal growth. Notably, a number of studies in humans exposed to an adverse environment in early life have demonstrated altered 5mC at the differentially methylated regions (DMRs) controlling the expression of the key fetal growth factor insulin like growth factor 2 (*IGF2*) and at the linked *H19* imprinting control region (*H19 ICR*). At birth, preterm infants had a significant decrease in 5mC at *DMR2* compared with term infants at birth ($\beta = -11.5$, $p < 0.001$) and compared with preterm infants at term

equivalent age (mean difference = -7.4, p = 0.01). By term equivalent age, preterm infants had decreased 5mC at both *DMR2* (β = –2.8, p = 0.01) and the *H19 ICR* (β = –2.3, p = 0.048) compared with term infants at birth, although this difference disappeared at 1 year corrected. Although research has suggested that catch up growth may confer an unfavourable metabolic phenotype, poor initial weight gain can associate with worse cognitive outcome. A pathway was established for obtaining advanced magnetic resonance images of the preterm brain. 5mC at *H19 ICR* and *DMR2* in buccal DNA showed no association with measures of white matter microstructure or whole brain volumes.

Term infants demonstrated telomere lengthening over the first year of life (mean difference = -0.3, p = 0.02). There was no significant change in telomere length over the first year of life in preterm infants (mean difference = 0.2, p = 0.34). However, as preterm infants at term equivalent age had longer telomeres compared to term infants at birth (β = 0.6, p < 0.001), ultimately there were no differences between the term and the preterm groups at 1 year corrected age (β = 0.3, p = 0.07).

The DNA modification 5-hydroxymethylcytosine (5hmC) is a stable modification in its own right and is also thought to be an intermediate step in DNA demethylation. 5hmC is abundant in the placenta but has not been studied in the context of fetal programming. Additionally, previous research using methods such as bisulphite conversion would not have discriminated between 5mC and 5hmC and therefore the role of 5mC may not have been accurately measured. To study the relationship between 5mC, 5hmC and fetal growth, gene expression of candidate imprinted and non-imprinted genes in full term placental samples from the Edinburgh Reproductive Tissue BioBank was analysed. 5mC and 5hmC within the *IGF2/H19* and *KvDMR* (controlling *CDKN1C*) loci was estimated using chemical capture and immunoprecipitation techniques that discriminate between modifications. Relationships between the expression of *IGF2* (r = 0.3, p = 0.02) and *CDKN1C* (r = -0.3, p = 0.01) and birth weight across the normal range were found and in keeping with the known action of these genes. 5mC at *IGF2 DMR0* (β = 0.3, p = 0.02) and *KvDMR* (β = 0.3, p = 0.02) and 5hmC at *H19* gene body (β = 0.2, p = 0.04) associated with birth weight.

Thus, DNA modifications at imprinted DMRs may modulate environmental influences on fetal growth across the normal range. DNA methylation at *IGF2/H19* can be influenced by early life events. It remains to be seen whether any changes are present later in childhood and whether they associate with risk factors for the metabolic syndrome.

Lay summary

Babies born prematurely are at greater risk of developing health problems and learning difficulties in later life than their full-term counterparts. This is very similar to findings in babies born small for dates compared with babies who were appropriately grown at full-term. The relationship between adversity early in life (being born early or small) and later disease is known as 'early life programming'. This may be caused by the initial slow growth followed by faster growth that preterm babies display, and also abnormal actions of stress hormone. Additionally, it has been proposed that one of the ways the early life environment might alter the disease risk is by altering the way that genes work. Potential mechanisms include changes to the normal chemical 'marks' that exist on our genes (known as epigenetic modifications) or how quickly the ends of our chromosomes (known as telomeres) shorten with age. Both can affect how genes function.

I studied 40 full-term and 46 preterm babies over their first year of life. The preterm babies remained, in general, smaller than the full-term babies, but by 1 year the differences were not as great. The preterm babies also had abnormal stress hormone levels in their saliva. The preterm babies showed differences in the epigenetic modifications and telomere length (taken from their saliva) soon after birth, but these differences had disappeared by 1 year of age. These epigenetic modifications did not show any relationship to measures of brain development taken by MRI scans.

Abnormalities in the placenta may also predispose to early life programming. I studied well-known genes in the placenta that are responsible for growth in the womb. Important epigenetic modifications at two of these genes varied with the weight of the baby, which may affect how these key genes work.

In summary, abnormal epigenetic modifications can be found in the placenta in babies born small and also in the saliva of babies born prematurely. We still need to see if these differences in epigenetic modifications early in life associate with health problems later in life.

Declaration

I declare that this thesis and the data presented within are a result of my own work carried out at the University of Edinburgh, with the following exceptions:

Dr Jessy Cartier performed Pyrosequencing for Chapters 3 and 4. Drs Devasuda Anblagan and Ahmed Serag did the image analyses for Chapter 4. Dr James Boardman did the statistical analysis for Chapter 4.

I declare that this work has not been previously submitted for any other degree or qualification.

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Publications and presentations

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Abbreviations

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADHD	Attention deficit hyperactivity disorder
AGA	Appropriate for gestational age
ASD	Autistic spectrum disorder
BMI	Body mass index
bp	Base pairs
BP	Blood pressure
BPD	Bronchopulmonary dysplasia
BWS	Beckwith–Wiedemann Syndrome
CDKNIC	Cyclin dependent kinase inhibitor
cDNA	Complementary DNA
CI	Confidence interval
CpG	Cytosine-phosphate-guanine
CRH	Corticotropin releasing hormone
CTCF	CCCTC binding factor
DEPCAT	Deprivation category
DEXA	Dual energy x-ray absorptiometry
DLK1	Delta like homolog 1

DMR	Differentially methylated region
dMRI	Diffusion MRI
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EDTA	Edetic acid
ERTBB	Edinburgh Reproductive Tissue BioBank
ES	Embryonic stem
FA	Fractional anisotropy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRB10	Growth factor receptor-bound protein 10
HOMA	Homeostasis model assessment
HPA	Hypothalamic-pituitary-adrenal
HSD11β2	11β hydroxysteroid dehydrogenase type 2
ICR	Imprinting control region
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IP	Immunoprecipitation
IQ	Intelligence quotient
IUGR	Intra-uterine growth restriction
KCNQ1	Potassium channel, voltage-gated, KQT-like subfamily, member 1

KCNQ1OT1	*Kcnq1* overlapping transcript 1
LGA	Large for gestational age
LINE-1	Long interspersed nuclear element-1
M	Molar
MBD	Methyl binding domain
MIQE	Minimum information for publication of quantitative real time PCR
MR	Magnetic resonance
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NHS	National health service
NR3C1	Nuclear receptor subfamily, group C, member 1
OFC	Occipito-frontal circumference
OMIM	Online Mendelian Inheritance in Man
PBS-BSA	Phosphate buffered saline – Bovine serum albumin
PCR	Polymerase chain reaction
PEG10	Paternally expressed gene 10
PHLDA2	Pleckstrin homology-like domain family A member 2
PMA	Post-menstrual age
PPARγ	Peroxisome proliferator-activated receptor-gamma
PVL	Periventricular leucomalacia

qPCR	Quantitative polymerase chain reaction
RCT	Randomised controlled trial
REC	Research Ethics Committee
RIN	RNA Integrity Number
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAM	*S*-adenosyl methionine
SD	Standard deviation
SDHA	Succinate dehydrogenase complex subunit A
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFT	Skin fold thickness
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
SRS	Silver–Russell Syndrome
TBE	Tris Boric acid EDTA
TBP	TATA box binding protein
TE	Tris EDTA
TET	Ten-eleven translocation
TL	Telomere length

TOI	Tract of interest
VLBW	Very low birth weight
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, zeta polypeptide
ZIM2	Zinc finger gene 2

Chapter 1: Introduction

1.1 Early life programming

There is substantial epidemiological evidence linking low birth weight to an increased risk of hypertension, type 2 diabetes mellitus, ischaemic heart disease, cerebrovascular accidents, obesity, cancer (Barker et al., 1989) (Barker et al., 1993) (Risnes et al., 2011) (Hales et al., 1991) (Oken & Gillman, 2003) (Lawlor et al., 2005), and neuro-psychiatric conditions (Cannon et al., 2002). The 'early life programming' or 'developmental origins of health and disease' concept that ensued, proposed that subjects exposed to adverse conditions during critical 'windows' of development, which may manifest as low birth weight, results in permanent changes to organ structure and function that are compensatory responses to maximise survival. These adaptations can be disadvantageous if the prenatal 'prediction' of the child/adult environment is incorrect as the ensuing reality is over-abundance (Barker, 1998) (Gillman, 2005). The typical example is of a fetus that was undernourished in utero assuming a 'thrifty phenotype', which is growth restriction but with relative brain sparing. The metabolic syndrome develops in later life following exposure to the effects of a plentiful extra-uterine environment (Hales & Barker, 2001). Similarly, it is feasible that preterm infants, whom are exposed to a suboptimal intra-uterine environment and thereafter withstand physiological stress and undernutrition ex-utero during a phase equivalent to the third trimester of pregnancy, would be at risk of early life programming.

The low birth weight individuals in historical epidemiological studies would have predominantly been full term infants born small for gestational age or growth restricted, however due to uncertainties regarding gestational age, would also have represented infants born preterm. Thus, the adverse outcomes reported in these studies may be associated either with fetal growth restriction or prematurity. Nevertheless, the relative contribution to the outcomes by preterm infants would have been small, given that few would have survived into adulthood to form the study cohorts. In contrast, the prognosis for very premature infants has improved dramatically due to advances in perinatal care in recent times and in resource rich

settings, survival to adulthood is routine. The incidence of preterm birth worldwide is increasing with an estimate of 11.1% of all births in 2010 (Blencowe et al., 2013), and the risk of silent complications of prematurity that this group carries forward is of growing concern.

I will describe the key age related conditions and their risk factors associated with preterm birth and the theoretical underlying biological mechanisms.

1.2 The burden of cardio-metabolic disease risk

1.2.1 Hypertension

There is now considerable evidence that prematurity is associated with raised blood pressure (BP) in young adulthood. Several studies have revealed that this relationship is independent of birth weight for gestation i.e. small for gestational age (SGA) and by extension, intra-uterine growth restriction (IUGR) (Irving et al., 2000) (Keijzer-Veen et al., 2005) (Hovi et al., 2007) (Rotteveel et al., 2008).

It is possible that in these studies, the sample sizes may have been too small to detect any additional risk IUGR may pose over and above prematurity. Indeed, the effect of IUGR as a risk factor was seen in a sample of 329 495 young men with gestational ages ranging from 24 to 43 weeks, but only amongst those born after 33 weeks gestation (Johansson et al., 2005). As late preterm births are more numerous than very preterm births, there is greater statistical power for an effect of growth restriction to be measured.. Whilst both earlier gestation and low birth weight are powerful predictors of morbidity and mortality, these two factors are not perfectly correlated owing to more variation in birth weight with increasing gestation. Within each interval of increasing gestational age, adverse outcomes increase exponentially as the birth weight percentile decreases (Kiely & Susser, 1992). Therefore the effect of fetal growth is more likely to be borne out amongst infants born close to or at full term and elements of the pathophysiology may differ from preterm infants. Also, prematurity may be a ‘double hit’ as preterm birth is often the consequence of a compromised pregnancy resulting in a degree of fetal growth restriction (Bukowski et al., 2001) (Lackman et al., 2001).

Nevertheless, these earlier studies have utilised single time point measurements of BP. In the largest population based study to date of 636 000 young adults born preterm, anti-hypertensive prescription was used as a surrogate measure for hypertension and the finding was that the prevalence increased with decreasing gestational age starting at 37 weeks gestation (Crump et al., 2011). Population studies such as these have been able to demonstrate that a dosage effect might exist according to the degree of prematurity (Johansson et al., 2005) (Crump et al., 2011). Additionally, adults born preterm have higher BP from ambulatory measurements (Kistner et al., 2005) but also higher individual BP variability, an independent risk factor of cardiovascular disease (Sipola-Leppänen et al., 2015).

A recent comprehensive systematic review and meta-analysis confirmed the estimation made in a previous review (de Jong et al., 2012) suggesting an increase of about 4 mmHg in systolic BP in preterm compared with term subjects and additionally that an increase in diastolic BP only occurs in women born preterm (Parkinson et al., 2013). This is significant when taking into account that lowering diastolic BP by as little as 2 mmHg can reduce overall morbidity: including the prevalence of hypertension by 17%, risk of coronary artery disease by 6% and risk of stroke and transient ischaemic attacks by 15% (Cook et al., 1995).

A few studies, but not all (Bonamy et al., 2005) (Bonamy et al., 2007), have suggested that preterm birth results in abnormalities to the vascular tree in childhood, adolescence and young adulthood, as indicated by raised carotid intima-media thickness (an early sign of atheroma formation) (Hovi et al., 2011) (Finken, et al., 2006) or impaired arterial elasticity (McEniery et al., 2011) (Rossi et al., 2011) or both (Lazdam et al., 2010), but sometimes only where there is also IUGR (Cheung et al., 2004) (Skilton et al., 2011). More recently, reduced capillary density was shown to contribute towards elevated BP (Lewandowski et al., 2015). These may mediate or even exacerbate the development of hypertension. Striking alterations in cardiac structure and function have been described by magnetic resonance imaging (MRI) in young adulthood, with increased left and right ventricular mass, but smaller right ventricle and impaired parameters of systolic and diastolic function. Systolic dysfunction of the right ventricle (which was worse than that of the left) and

increased left ventricular mass index are important predictors for cardiovascular morbidity and mortality. The severity of the changes was graded along with the degree of prematurity, again highlighting a dosage effect. These changes were independent of microvascular abnormalities and BP (Lewandowski et al., 2013a) (Lewandowski et al., 2013b) (Lewandowski et al., 2015).

1.2.2 Insulin resistance/ type 2 diabetes

Of the components of the metabolic syndrome, insulin resistance and associated hyperinsulinism has been recognised to be an early manifestation amongst individuals born SGA and is a precursor of type 2 diabetes (Reaven, 1993). A recent systematic review concluded that preterm birth is associated with insulin resistance throughout the life course, however individual studies have yielded conflicting evidence, particularly in adulthood, due to their heterogeneity (Tinnion et al., 2014).

In childhood, prematurity associates with insulin resistance in children as young as 4 years of age (Hofman et al., 2004) with up to 50% lower insulin sensitivity (Regan et al., 2006). Of note, the indices were of similar magnitude in children born SGA at term. Gestational age at birth associated inversely with elevated plasma insulin levels in a prospective study of 1358 subjects at birth and at a median age of 1.4 years, indicating that insulin resistance may be present from very early in life (Wang et al., 2014).

There were no significant differences in fasting glucose or insulin concentrations between adults (at a mean age of 19.6 years) born preterm and at term in a large systematic review and meta-analysis, although the authors comment that more robust assessments such as homeostasis model assessment (HOMA) are wanting (Parkinson et al., 2013). Hovi et al demonstrated that young adults born very low birth weight (VLBW) had higher fasting insulin and, in response to an oral glucose tolerance test, higher 2 hour insulin, 2 hour glucose concentration and HOMA index versus controls (Hovi et al., 2007). Insulin resistance was also observed using the clamp design, considered the gold standard for measuring insulin sensitivity (Rotteveel et al., 2008). No significant differences were observed between individuals born SGA or appropriate for gestational age (AGA) in all of these studies. However, this is in

contrast to some authors who argued that SGA was an independent determinant of insulin sensitivity and secretion (Bazaes et al., 2004) (Fewtrell et al., 2000).

Two large retrospective cohort studies showed a relationship between prematurity and a medical diagnosis of type 2 diabetes mellitus (Lawlor et al., 2006) (Kaijser et al., 2009), however these are historical cohorts and most very preterm individuals surviving due to modern neonatal intensive care have yet to reach high-risk age. As with raised BP, small differences in glucose homeostasis detected in young adulthood could translate into clinical significance later in life and the degree of fat mass is a potent modifier of risk (Finken, et al., 2006) (Tinnion et al., 2014).

1.2.3 Altered adiposity

Several techniques have been used to quantify the adiposity of preterm infants at term corrected age, chiefly MRI, air displacement plethysmography and dual energy x-ray absorptiometry (DEXA). They individually yield mixed results as they all are indirect techniques and require prior assumptions (Ellis, 2007). However, a meta-analysis concluded that preterm infants have a 3% greater percentage fat mass at term corrected age compared with full term infants and that this appears to be attributed to the reduced acquisition of lean tissue rather than excess fat mass (Johnson et al., 2012). This pattern, with decreased weight, relative greater adiposity, and most importantly, a deficit in lean tissue, echoes that which has been observed in full-term Indian babies ('thin-fat'), where it has been associated with an increased risk of type 2 diabetes and relative adiposity in later life (Yajnik et al., 2003). Compared to SGA full term infants at birth, preterm infants who were SGA had greater percentage fat mass by term corrected age (Giannì et al., 2009) suggesting that prematurity may be a greater risk for altered adiposity.

MRI allows not only quantification of total adiposity, but also detailed assessment of partitioning. Whilst preterm infants at term corrected age had no difference in total adiposity compared to term-born infants at birth, fat distribution may differ: for example one study showed that preterm infants have less subcutaneous fat and more intra-abdominal fat compared to term infants (Uthaya et al., 2005). The severity of illness was related to the volume of intra-abdominal fat, which led the authors to

speculate that the observed pattern of fat deposition may be due to the raised levels of endogenous cortisol that preterm infants are thought to have, similar to the pathogenesis of Cushing's syndrome (Uthaya et al., 2005). Furthermore, preterm infants had raised intra-hepatocellular fat at term age, similar to that seen in adults with visceral adiposity (Thomas et al., 2008).

Percentage body fat measurements by densitometry approximate those of term born babies by 3 to 4 months corrected age (Ramel et al., 2011), though during childhood, ex-preterm infants have a lower body mass index (BMI) than term born counterparts (Hack et al., 2003). However, the pattern of fat deposits favours the trunk relative to the extremities (Giannì et al., 2008). Thereafter, there may be a greater change in BMI z-score between childhood and adolescence such that the rate of obesity in ex-preterm adolescents mirrors that of their term born counterparts (Hack et al., 2011). Although the BMI of ex-preterm individuals may remain equivalent to that of term born individuals in young adulthood (Parkinson et al., 2013), the aberrant distribution of adiposity and increased intra-hepatocellular fat noted at term corrected age may persist, along with an increase in total body fat (Thomas et al., 2011). These are recognised components of the metabolic syndrome and probably related to the rapid change in BMI between childhood and adolescence. There were similar findings of increased total body fat as measured by DEXA, which was inversely proportional to gestational age (Breukhoven et al., 2012). This finding, given that the cohort was relatively mature (average gestational age of 32 weeks) and infants with serious neonatal complications were excluded, is significant. Whether this altered body composition persists into mature adulthood remains to be seen.

1.3 Potential mechanisms

1.3.1 Catch-up growth

Currently used preterm growth charts are based on cross-sectional data using birth weights of infants born preterm and term, so that preterm infants are expected to achieve the weight of a term newborn by their expected date of delivery. Further complicating the matter is that the birth weight of an average preterm infant reflects the end of a compromised pregnancy and may be less than that of a healthy fetus

(Bukowski et al., 2001) (Lackman et al., 2001). A recent UK population wide study has shown that, in reality, infants born <32 weeks gestation dropped two percentile channel widths to reach a steady state of growth between the 0.4th to 9th percentile during their hospital stay (Cole et al., 2014). This reflects the nutritional management and other aspects of intensive care practised in recent years, where difficulties with enteral and parenteral nutrition, increased metabolic demands and illness can lead to a slower rate of weight gain than in fetal life and a divergence away from the birth percentile. Because preterm infants do not mimic normal intra-uterine growth patterns during the equivalent period, they may resemble the thrifty phenotype of the growth restricted term newborn (Hales & Barker, 2001). Here, compensatory changes that occurred in 'fetal' life (during hospitalisation) that would be predicted to enable survival are mismatched with the postnatal environment (post-discharge) of nutritional abundance.

This period of slower growth restriction sets the scene for what is described as catch-up growth (Karlberg & Albertsson-Wikland, 1995). There is evidence that catch-up growth confers additional risk for the metabolic syndrome or may be an independent risk factor. A systematic review of 80 studies from 1996 to 2000 reported that both birth weight and head circumference at birth were inversely related to systolic blood pressure and also, that accelerated postnatal growth was associated with raised blood pressure (Huxley et al., 2000). Low birth weight, thinness in infancy and an upward trajectory in BMI in childhood associated with insulin resistance and coronary events (Barker et al., 2005). Similarly, in a study of Indian children: gain in BMI in childhood without becoming overweight led to impaired glucose tolerance, diabetes and obesity in adulthood (Bhargava et al., 2004). The accretion of fat mass, being a major determinant of insulin sensitivity, may explain this association (Hyppönen et al., 2003). As in growth restricted term infants, preterm infants have demonstrated catch-up growth during infancy, childhood and adolescence (Hack et al., 2003). Specifically, increased height and weight gain in childhood have been linked with insulin sensitivity and blood pressure (Fewtrell et al., 2000) (Rotteveel et al., 2008) and increase in BMI and fat distribution (Euser et al., 2005).

Follow on studies of early randomised controlled trials (RCTs) of nutritional interventions in neonatal care suggested that lower nutrient intake and slower initial growth was associated with a reduced risk of insulin resistance (Singhal et al., 2003) and that the use of donor breast milk, as opposed to preterm formula, associated with lower blood pressure in adolescence (Singhal et al., 2001). Therefore the authors proposed that the causative factor in metabolic programming was the phenomenon of postnatal growth acceleration in both growth restricted term infants and preterm infants, independent of birth weight or gestation (Singhal & Lucas, 2004). Further studies showing that infants fed a nutrient enriched formula promoting weight gain had higher blood pressure than infants fed standard formula supported this argument (Singhal et al., 2007). Additional arguments against promoting rapid weight gain have come from animal studies of low or normal birth weight where dietary interventions to slow postnatal growth increase longevity and protect against the risks posed by a high carbohydrate, high fat diet in later life (Ozanne & Hales, 2004) (Jimenez-Chillaron et al., 2006).

However, in preterm infants, any potential benefits of slow postnatal growth must be weighed against the deleterious effects of undernutrition on the developing brain. These appear to be competing outcomes with a complex trade-off to be made between avoiding adverse metabolic outcomes, through slower weight gain, and optimising brain development, by increasing weight gain. Indeed, faster weight gain in infancy associated with better neurodevelopmental outcome in childhood and with an increase in blood pressure, albeit modest (Belfort et al., 2010). Preterm infants with faster weight gain in hospital and in the first 2 years show better neurodevelopmental outcomes at 2 years (Ehrenkranz, 2006) (Latal-Hajnal et al., 2003). Growth rate is related to nutritional intake (Ehrenkranz et al., 2011). Higher protein and energy intake in the first week after birth associated with better developmental outcomes at 18 months (Stephens et al., 2009). These are nevertheless observational studies and illness severity associates with reduced growth and poorer outcomes. In post hoc mediation analyses of prospectively collected data following a glutamine supplementation trial, total (parenteral and enteral) energy intake in the first week was a significant mediator of the relationship between illness severity and

later growth and adverse outcomes (Ehrenkranz et al., 2001). Further post hoc studies in babies who had taken part in those early RCTs of nutritional intervention showed that nutrient enriched formula intake associated with larger caudate volumes and better cognition in adolescence (Isaacs et al., 2008). Besides the early trials showing that standard infant formula led to worse cognitive scores in childhood when compared with nutrient enriched preterm formula (Lucas et al., 1998), there is little experimental data showing that nutritional regimens aiming to achieve more rapid growth lead to improved neurodevelopmental outcome. In a contemporaneous RCT, higher parenteral nutritional intake (protein and total calorie) in very preterm infants benefited head growth as measured by occipito-frontal circumference (OFC) as the primary outcome and data on neurodevelopmental outcome is awaited (Morgan et al., 2014).

Whilst acknowledging that studies on the effects of breast milk are confounded by factors that affect the mother's choice to breastfeed or not, such as maternal education and socio-economic status, several lines of evidence indicate that neurocognitive outcomes are better in term and preterm breast milk fed infants (Vohr et al., 2006) despite slower growth (Dewey, 1998). Breast milk intake in hospital favoured tests of intelligence quotient (IQ), brain volume and white matter development in childhood in a dose-response manner (Isaacs et al., 2010). Also, the extent of breast milk exposure predicted the degree of head growth in the neonatal period despite a decrease in standard deviation scores for weight (Cockerill et al., 2006). More recently, studies in two independent cohorts of very preterm infants suggested that breast feeding at the time of discharge home is associated with better neurodevelopment despite poorer weight gain in hospital (Roze et al., 2012).

In summary, it is not known which growth pattern in the neonatal period is ideal for very preterm infants in the long-term with respect to both metabolic health and intact neurodevelopment. That the rate of growth should approximate that of the healthy fetus is unproven and, based on studies above, may be unfavourable. Furthermore, weight gain is a poor indicator of the quality of growth, as it does not reflect changes in body composition.

1.3.2 Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis

Fetal overexposure to glucocorticoids may also lead to early life programming (Edwards et al., 1993). In animal studies, maternal administration of glucocorticoids led to low birth weight, raised blood pressure (Benediktsson et al., 1993), insulin resistance and also altered HPA axis activity with some gender specific differences (Sloboda et al., 2002) (O'Regan et al., 2004). It is thought that this alteration in the HPA axis may be one mechanism that contributes to the development of cardio-metabolic disease by changing the trajectory of maturation of organ systems (Edwards et al., 1993). Supporting this are several population studies of adults born small where elevated fasting plasma cortisol levels or cortisol reactivity associated with cardiovascular risk factors such as raised blood pressure, glucose intolerance and dyslipidaemia (Phillips et al., 1998) (Phillips et al., 2000) (Reynolds et al., 2001). Moreover, a meta-analysis showed an inverse relationship between circulating cortisol levels and birth weight (van Montfoort et al., 2005). Early morning fasting cortisol levels representing a 'stressed' state rather than basal levels associated with cardiovascular risk factors, thus linking maladaptation to early life stress (Reynolds, 2013b). Additionally, the neurological sequelae associated with low birth weight may be attributable to HPA axis activation given the concomitant reduction in fetal brain growth (Huang et al., 1999) and function (Aghajafari et al., 2002), delay in myelination (Dunlop et al., 1997), and behaviour reminiscent of anxiety (Welberg et al., 2001) observed in animals following maternal glucocorticoid administration.

Antenatal glucocorticoids, in the form of synthetic betamethasone or dexamethasone, are crucial in reducing mortality and morbidity in preterm infants born at less than 35 weeks gestation and in some instances repeated courses may be administered (McKinlay et al., 2012) but the latter remains controversial. The fetus is generally protected from excess maternal glucocorticoids by the action of the type 2 isoform of 11β hydroxysteroid dehydrogenase (HSD11β2) in the placenta, which converts active cortisol to inactive cortisone. This 'barrier' effect is not complete as maternal glucocorticoids are essential for normal development and maturation of fetal organs (Benediktsson et al., 1997). Betamethasone and dexamethasone are not inactivated

by HSD11β2 and thus freely cross the placenta to exert their intended effects on the fetus (Blanford & Murphy, 1977).

Preterm infants who have been exposed to antenatal glucocorticoids have altered stress reactivity, according to a systematic review of 49 studies: suppression of endogenous cortisol production after birth seems to recover by 2 weeks of age and thereafter, a blunted response to pain was observed, persisting throughout the first 4 months of life. This response was inversely proportional to the number of courses of antenatal glucocorticoids or the total dose administered (Tegethoff et al., 2009). Cortisol responses to corticotropin releasing hormone (CRH) stimulation in a group of preterm infants <30 weeks gestation were significantly lower at 2 weeks of age compared to term equivalent age, and the responses to CRH were further blunted in those exposed to antenatal glucocorticoids (Niwa et al., 2013). In the longer term, there appears to be a switch from low levels of cortisol (at 3 months) to elevated levels at 8 and 18 months in preterm infants in general compared with full term infants (Grunau et al., 2007).

Observational and experimental studies suggest that antenatal glucocorticoid exposure has some long-term adverse clinical effects, both metabolic and neurological. Exposure to a single course of antenatal betamethasone was associated with higher systolic and diastolic blood pressures in adolescence (Doyle et al., 2000). However, this finding was not mirrored in an separate study of 19 year old individuals born preterm (Finken et al., 2008). Infants from the Auckland Steroid Trial – whose mothers were randomised to receive either betamethasone or placebo - were examined at 30 years of age. Whilst there were no effects on body size, hypertension, diabetes or cortisol levels, there were indications of insulin resistance, particularly in women (Dalziel et al., 2005b). There were no indicators of cognitive impairment or psychiatric symptoms (Dalziel et al., 2005a).

A recent systematic review suggested consideration of repeated doses of glucocorticoids to reduce neonatal morbidity, and whilst there were no demonstrable benefits or significant harm in very early childhood (McKinlay et al., 2012), some findings cause concern. Follow-up of children in the Australasian Collaborative Trial

of Repeat Doses of Steroids reported no differences in either BP or body size at 2 years, however those exposed to repeat doses were more likely than the placebo group to have problems with attention (Crowther et al., 2007). The similar National Institute of Child Health and Human Development (NICHD) trial of repeated dosing also showed no difference in anthropometry, BP or scores of cognitive or motor development at 30 months, but although not statistically significant, there were more cases of cerebral palsy in the repeated dosing group (Wapner et al., 2007). In an observational study, increasing numbers of courses of betamethasone was associated with increased aggression, distractibility and hyperkinetic behaviour in children at both 3 and 6 years of age (French et al., 2004). Surprisingly, a reduction in cerebral palsy was noted (French et al., 2004), despite a reduction in birth weight and head circumference (French et al., 1999).

Whether preterm infants have altered HPA axis activity due to antenatal steroids per se is difficult to ascertain. Various aspects of neonatal intensive care may confound the effect: the number of painful procedures during neonatal intensive care predicted higher cortisol secretion (Grunau et al., 2004), as did earlier gestational age at birth (Grunau et al., 2004) (Niwa et al., 2013), both possibly related to illness severity (Ng et al., 2011). Glucocorticoids are occasionally used postnatally to treat hypotension or to aid in reducing respiratory support in bronchopulmonary dysplasia and may further affect cortisol homeostasis. Ultimately, preterm infants have higher levels of plasma cortisol in the first 4 weeks of life than fetuses of equivalent gestations who have the advantage of the placental barrier (Glover et al., 2005). To address this confounding effect of prematurity, Alexander et al studied 6-10 year old children who were born at term, but were exposed to glucocorticoids during gestation to promote fetal maturation. Compared to controls, these children had increased cortisol reactivity to a standardised laboratory stressor (Alexander et al., 2012). Maternal stress can itself exert an effect on the developing HPA axis, brain morphology and behaviour (Buss et al., 2012) and the study addressed this additional confounding factor by including a group of children whose mothers were hospitalised during pregnancy and did not receive glucocorticoids (Alexander et al., 2012). More recently, a large population study which robustly adjusted for confounders by design

and analysis identified a link between antenatal glucocorticoid exposure and symptoms of attention deficit hyperactivity disorder (ADHD) in childhood and adolescence (Khalife et al., 2013). The mechanisms may include thinning of the rostral anterior cingulate cortex which associated with affective symptoms observed in term born children exposed to betamethasone compared to controls (Davis et al., 2013).

The unequivocal benefits of antenatal glucocorticoids outweigh the small risks of HPA axis alteration, but this in addition to stressors associated with preterm birth may be a mechanism of disease programming. As discussed below, potential molecular mediators may be epigenetic modifications or telomere attrition.

1.3.3 Epigenetics

The term 'epigenetics' is commonly used to describe alterations in gene function without changes to the DNA sequence. Epigenetic processes are not only essential for the development of cellular identity and normal development (Li et al., 1992), but might also mediate the interaction of the environment with the genome (Jaenisch & Bird, 2003). Alterations in the epigenome may be an important mechanism linking factors in the early life environment (such as nutrition and stress) with later disease risk (Jirtle & Skinner, 2007).

Changes to the epigenome occur via several molecular modifications such as DNA methylation, histone modifications and non-coding RNAs. Of these, DNA methylation is considered to be the main component and is the best characterised. It is also considered the most stable and accessible epigenetic mark for quantitative measurements in human populations. DNA methylation is where the addition of a methyl group at the 5-position of a cytosine base forms 5-methylcytosine (5mC) through the action of the DNA methyltransferase enzymes (DNMTs), which use *S*-adenosyl methionine (SAM) as a methyl donor (Figure 1.1). DNA methylation mainly occurs at cytosine bases paired with guanine by a phosphate residue - known as cytosine-phosphate-guanine (CpG) dinucleotides. In general, when DNA methylation occurs at gene promoters, it tends to be associated with recruitment of methyl-CpG-binding proteins, gene silencing and heterochromatin formation. DNA

methylation is also responsible for the silencing of repetitive elements and X chromosome inactivation (Bird, 2002).

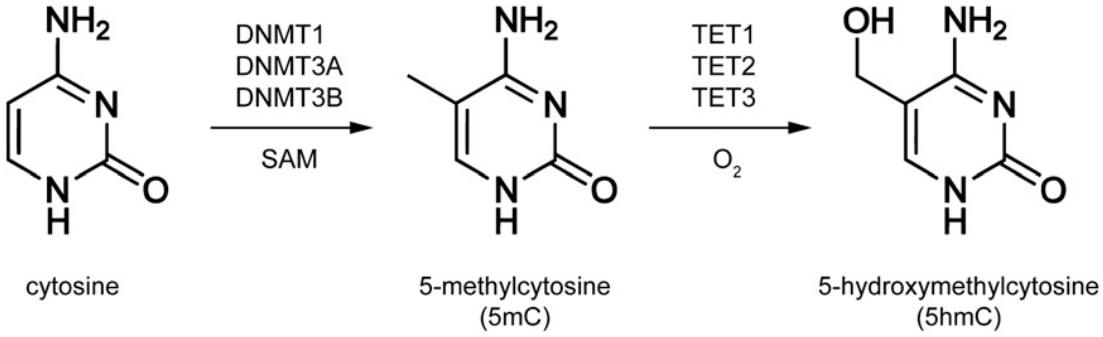


Figure 1.1 Unmodified cytosine, 5mC and 5hmC. 5mC is formed by the addition of a methyl group to cytosine by the action of DNA methyltransferases (DNMTs), which uses *S*-adenosyl methionine (SAM) as a methyl donor. 5hmC is formed though the action of TET proteins, which uses oxygen to transfer a hydroxyl group to 5mC. Figure obtained from Dahl et al (Dahl et al., 2011).

Patterns of DNA methylation across the genome are conserved during mitosis during life, but are removed from somatic and germ cell lines and reset during embryonic development, in tissue specific patterns (Jaenisch & Bird, 2003). This is a critical period where alterations in this process could have long-term effects. Notably, methylation marks at imprinted genes are maintained during the post fertilisation period of epigenetic reprogramming, but data is emerging that other regions are also resistant to reprogramming (Radford et al., 2014).

1.3.3.1 Genomic imprinting

Genomic imprinting is the phenomenon whereby genes are expressed from one allele according to its parental origin (McGrath & Solter, 1984). In many cases, the silencing of the inactive allele is achieved by DNA methylation marks that are laid down at differentially methylated regions (DMRs) (Li et al., 1993). During gametogenesis in the new organism, these sex-specific imprint marks are erased and re-established in the germ line. They are known as primary DMRs and these methylation marks are maintained post-fertilisation in somatic cells undergoing organogenesis (Reik & Walter, 2001). 'Secondary' DMRs are established after

fertilisation as a consequence of DNA methylation at primary DMRs and contribute to stable mono-allelic expression. Most imprinted genes are found in clusters and share a common primary DMR regulating the imprinting of multiple genes in the cluster (Edwards & Ferguson-Smith, 2007).

A large cluster of imprinted genes, which is located at chromosome 11p15.5, is organised into two regulatory domains, each with a specific imprinting control region (ICR) and contains several genes important for growth and metabolism of which the key candidates are shown in Figure 1.2.

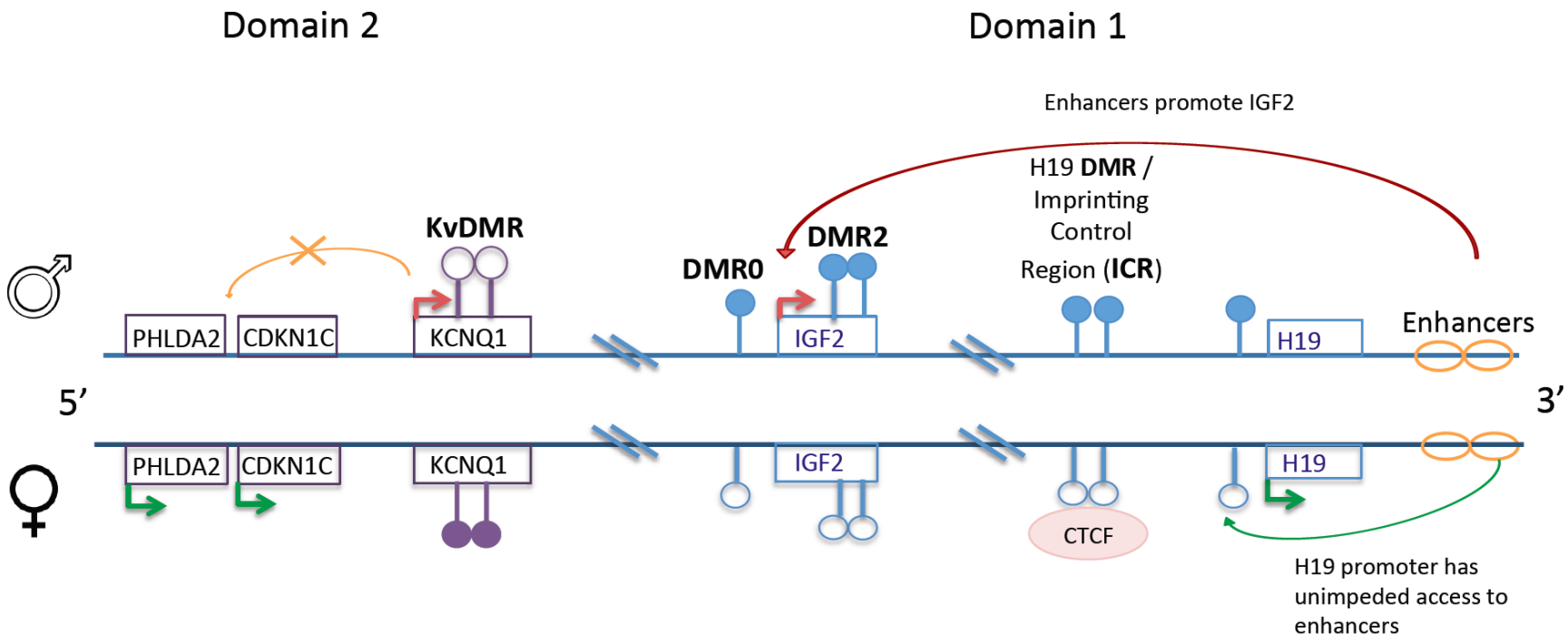


Figure 1.2 The imprinted cluster on chromosome 11p15.5

The telomeric, 3' prime domain includes the Insulin like growth factor (*IGF2)* and *H19* genes. The centromeric, 5' domain includes *CDKN1C*, encoding a cyclin dependent kinase inhibitor, *PHLDA2*, pleckstrin homology-like domain family A member 2 and the potassium channel, voltage-gated, KQT-like subfamily, member 1 (*KCNQ1)* gene. The unfilled and filled 'lollipops'represent unmethylated and methylated regions respectively. The arrows with elbows indicate transcription.

The telomeric domain includes the Insulin like growth factor 2 (*IGF2*) and *H19* genes which are oppositely imprinted (DeChiara et al., 1991) (Bartolomei et al., 1991). Allele-specific expression is regulated by DNA methylation at the *H19* promoter and three DMRs - *DMR0* and *DMR2* proximal to the *IGF2* gene and the ICR upstream of *H19* (Phillips & Corces, 2009). The *H19 ICR* contains 7 binding sites for the methylation sensitive, zinc finger protein CCCTC binding factor (CTCF). CTCF binds to the unmethylated maternal allele and facilitates the assembly of a chromatin insulator, preventing the *IGF2* promoter from accessing the enhancers downstream, resulting in silencing of *IGF2* and transcription of *H19*. On the paternal allele, CTCF binding (and insulator assembly) is blocked by DNA methylation allowing functional communication between promoters and enhancers which drive *IGF2* expression (Kurukuti et al., 2006) (Phillips & Corces, 2009).

In mouse knock out models, *Igf2* has been shown to be the major driver of prenatal growth (DeChiara et al., 1990) by its action on the diffusional exchange capacity of the placenta (Constância et al., 2002) (Sibley et al., 2004). *H19* transcribes a large non-coding RNA (Brannan et al., 1990) and its function is unclear although evidence suggests it may function as a tumour suppressor (Hao et al., 1993) (Yoshimizu et al., 2008) which may account for its action to restrict growth. In mice, targeted deletions of *H19* yield an overgrowth phenotype and conversely, *H19* overexpression result in growth restriction (Gabory et al., 2009). Further data has shown that *H19* transcribes a microRNA that is expressed exclusively in placenta and can suppress genes promoting placental growth (Keniry et al., 2012). More recently, *H19* is thought to interact with methyl-CpG-binding domain protein 1 which maintains repressive histone marks on 9 targets of an Imprinted Gene Network (Monnier et al., 2013). In humans, classical examples of altered expression of imprinted genes leading to growth abnormalities are the disorder of overgrowth, Beckwith–Wiedemann Syndrome (BWS, Online Mendelian Inheritance in Man (OMIM) 130650), and the growth restriction disorder, Silver–Russell Syndrome (SRS, OMIM 180860), where opposite alterations in DNA methylation at DMRs controlling *IGF2* have been reported (Reik et al., 1995) (Eggermann et al., 2008) (Murrell et al., 2008).

The larger centromeric domain of 11p15.5 includes several growth-related genes, such as *CDKN1C*, encoding a cyclin dependent kinase inhibitor, and *PHLDA2*, pleckstrin homology-like domain family A member 2, both major negative regulators of embryonic growth (Andrews et al., 2007) and extra-embryonic growth (Tunster et al., 2010). Both genes are expressed from the maternal chromosome (Matsuoka et al., 1996). The ICR, *KvDMR* (also known as *ICR2*) corresponds to the promoter of the potassium channel, voltage-gated, KQT-like subfamily, member 1 (*KCNQ1)* gene, whose non-coding RNA, *KCNQ1* overlapping transcript 1 (*KCNQ1OT1)*, is normally expressed only from the paternal chromosome and elongation silences the adjacent imprinted genes (Mancini-DiNardo, 2003). Loss of *KvDMR* methylation on the maternal chromosome, resulting in *KCNQ1OT1* activation and bi-allelic *CDKN1C* silencing (Diaz-Meyer et al., 2003), is the most frequent cause of BWS (Lee et al., 1999) (Smilinich et al., 1999). On the other hand, *CDKN1C* and *PHLDA2* overexpression have been found in cases of fetal growth restriction and demise where there have been mutations in *KCNQ1* resulting in lack of function (De Crescenzo et al., 2013). Although duplications of the *KvDMR* domain result in a SRS-like phenotype (Schönherr et al., 2007), in the vast majority of SRS, abnormal DNA methylation (hypomethylation) occurs at the *H19 ICR* and promoter (Netchine et al., 2007) (Azzi et al., 2015).

There are further interesting aspects of imprinted genes. ICRs are known to repress genes by utilising one of two mechanisms: transcription of a non-coding RNA or by CTCF mediated insulation of enhancers (Lewis & Reik, 2006). The former is usually on a maternally methylated region (such as *KvDMR*) and contains the promoter for an anti-sense transcript, the latter is usually on a paternally methylated region, does not contain a promoter and is inter-genic (such as *H19 ICR*) (Edwards & Ferguson-Smith, 2007) Both *KvDMR* and *H19 ICR* are primary (gametic) regions (Edwards & Ferguson-Smith, 2007) and, as mentioned above, aberrant imprinting can result in BWS or SRS. So, whilst they function differently, they result in similar phenotypes.

According to the parental ‘conflict’ or ‘kinship’ theory proposed by Moore and Haig, imprinted genes evolved as a result of competition between the maternal and paternal genomes over provision of maternal nutrients to the developing fetus (Moore &

Haig, 1991). They predicted, correctly, that in general, paternally imprinted (expressed) genes act to increase fetal growth by promoting acquisition of maternal resources and conversely, maternally imprinted (expressed) genes act to restrict fetal growth. (The leading example is that of *IGF2* and the oppositely imprinted *IGF2* receptor (*IGF2R*) which acts to degrade IGF2 (Haig & Graham, 1991), however the latter is imprinted in mouse and not in human (Tycko & Morison, 2002).) This occurs since in many mammalian species, an offspring's paternally derived genes are less likely to be present in the mother's other offspring, therefore they have less to lose from the costs to the mother's future reproduction. Moore and Haig also theorised that imprinted genes would further affect how much an offspring would extract from its mother at the expense of its siblings after birth. As such, imprinted genes would be involved in suckling, appetite, postnatal metabolism, behaviour and growth (Moore & Haig, 1991). These characteristics are typified by the phenotypes of the syndromes already mentioned, BWS and SRS, but also Prader-Willi (OMIM 176270) and Angelman syndromes (OMIM 105830) (Nicholls, 1993) (Haig & Wharton, 2003). Indeed, studies of several imprinted genes support the kinship theory (Tycko & Morison, 2002).

The kinship theory further predicted that imprinting would have an important role in the development of the placenta – the key conduit of limited nutrients to the embryo (Reik et al., 2003). Indeed, it is believed that imprinting evolved along with placentation. Imprinting is absent in the egg-laying platypus, which displays both mammalian and reptilian features, and belongs to the lineage preceding the later divergence of marsupials and eutherian mammals (John & Surani, 2000). Marsupial fetal development is short lived, the placenta more rudimentary and the majority of support for growth of the offspring occurs during an extended and complex period of lactation. Despite this, imprinting is present and much is conserved with eutherian mammals (John & Surani, 2000).

In this regard, imprinted genes are of particular interest as they are developmentally regulated by DNA methylation, important for growth and development, abundant in the placenta and aberrations acquired in early life can have consequences later on. Despite *IGF2*/*H19* being a classically imprinted locus, evidence indicates that

variation or heterogeneity in DNA methylation can occur in the normal population (Rancourt et al., 2013) as a result of heritable, environmental and stochastic factors (related to inaccuracies of the machinery maintaining epigenetic patterns) and may be a normally distributed trait (Heijmans et al., 2007).

1.3.3.2 DNA methylation in fetal growth and preterm birth

Experimental studies have shown that transient environmental influences can cause persistent changes to the epigenome and can 'programme' adult disease. Data in humans have been limited to cross-sectional or quasi-experimental studies.

The mechanism(s) underlying increased disease risk in infants exposed to excessive glucocorticoids may include altered DNA methylation. This can be seen in the dexamethasone programmed rat model. The growth restricted offspring that would have gone on to develop glucose intolerance (Nyirenda et al., 1998) were found to have altered DNA methylation of *Igf2* in liver in association with altered gene expression (Drake et al., 2011). Glucocorticoids have been shown to cause demethylation of specific fetal hepatic gene promoters in late gestation (Thomassin et al., 2001). Endogenous glucocorticoids also appear to exert an effect on DNA methylation: maternal stress/anxiety during pregnancy is associated with increased DNA methylation of the promoter of the glucocorticoid receptor – nuclear receptor subfamily, group C, member 1 (*NR3C1*) (implying reduced transcription and glucocorticoid receptor activity) in umbilical cord blood cells and a heightened cortisol response to stress in the infant (supposedly from deficient negative feedback) (Oberlander et al., 2008). Deficient early care giving behavior in rodents caused increased DNA methylation at the hippocampal *NR3C1* promoter (Weaver et al., 2004). Translating this finding: increased DNA methylation of the *NR3C1* promoter (and reduced transcript levels) in the hippocampus of suicide victims with a history of childhood abuse was described (McGowan et al., 2009). The impact of early life stress may extend more broadly on a genome wide scale, as measured in adolescence (Essex et al., 2013).

Stressors accompanying low socio-economic status can also have a bearing on DNA methylation. Early life socio-economic status appeared to embed into DNA

methylation as measured in adult life such that DNA methylation was a greater predictor than current socio-economic status (Borghol et al., 2012) and was also related to cortisol secretion and perceived stress (Lam et al., 2012). A component of low socio-economic status or perhaps compounding it is exposure to cigarette smoking. Alterations in DNA methylation can be seen as early as in fetal life at the candidate genes *IGF2* and *NR3C1* (Drake et al., 2015). In umbilical cord blood, changes at *IGF2* (Murphy et al., 2012) and genome wide (Joubert et al., 2012) have been described. The sensitivity of the methylome to prenatal maternal smoking appears to persist into childhood and the late teens (Richmond et al., 2015).

Early nutrition could also influence the adult phenotype via DNA methylation. Dietary methyl donors and cofactors (such as B12, folate, pyridoxine and methionine) are necessary for the synthesis of *S*-adenosyl methionine, required for establishing and maintaining methylation during development. Restriction of B12, folate and methionine at clinically relevant levels around the time of conception resulted in adulthood hypertension, insulin resistance, adiposity and widespread changes to the epigenome in sheep (Sinclair et al., 2007). In humans, children whose mothers took peri-conceptional folic acid at the recommended dosage had higher methylation at *IGF2 DMR0* compared to those whose mothers did not (Steegers-Theunissen et al., 2009). Folic acid during pregnancy may also contribute to DNA methylation at *IGF2*, but the direction of change was not consistent (Hoyo et al., 2011). The European Society of Paediatric Gastroenterology, Hepatology and Nutrition recommend that preterm infants should receive folic acid supplementation (Agostoni et al., 2010). Although evidence is sparse, due to supplementation in parenteral nutrition and breast milk fortifiers, it has not been shown that very preterm infants are at risk of becoming folate deficient unless their sole nutrition has been unsupplemented human milk, born to mothers who smoked or mothers with prior folate deficiency (Oncel et al., 2014).

A reduction in methylation was seen at *IGF2 DMR0* in peripheral blood of adults who were conceived during the Dutch Hunger Winter of 1944-1945 (a period of severe, acute malnutrition) (Heijmans et al., 2008). A similar decrease in methylation was seen at neighbouring DMRs at the *IGF2* locus such as *DMR2*, but not the *H19*

ICR (Tobi et al., 2012). Further candidate genes involved in metabolism, growth and cardiovascular disease also showed alterations in DNA methylation (Tobi et al., 2009) and from an unbiased and genome wide approach, changes were found to be preferentially at regulatory regions mapping to genes enriched for differential expression during early development, and in pathways involved in metabolism and growth (Tobi et al., 2014). Whilst changes were measurable if famine occurred at any time during gestation, the greatest effect was exposure in the first 10 weeks suggesting that critical windows of programming exist (Tobi et al., 2015), the timing of which may depend on the specific insult and the outcome. In a similar vein, the offspring of mothers who consumed a harsh, unbalanced diet during pregnancy in Motherwell, Scotland, had altered DNA methylation at the *H19 ICR, NR3C1* and *HSD11β2* in peripheral blood in adulthood (Drake et al., 2012).

It is remarkable that changes in the methylome can be detected several decades after the sentinel event further suggesting that they are stable alterations. These changes may be causal in the increased risk of obesity, type 2 diabetes, dyslipidaemia and schizophrenia observed in the Dutch cohort (Lumey et al., 2011) and hypertension and altered HPA axis activity in the Motherwell cohort (Drake et al., 2012). Additionally, malnutrition, as severe as in these studies, may also evoke a stress response on the mother and fetus and alter the epigenome or mediate the phenotypic outcomes. However, confounding by postnatal and life-course factors are highly possible and it is also plausible that changes in DNA methylation might arise as a consequence of the disease state rather than be causative, hence the importance of undertaking longitudinal studies from early life.

Studies of early life in humans have been cross-sectional showing associations between DNA methylation and variation in birth weight. Accessible tissues such as cord blood, umbilical cord, but mostly placenta were used and either targeted specific genes implicated in growth or metabolism, or were methylome wide. Notable was the methylome wide study in cord blood from IUGR and AGA subjects. The finding of altered DNA methylation at the promoter of the nuclear transcription factor hepatic nuclear factor 4α (*HNF4α*) was important as mutations in *HNF4α* is linked to diabetes in later life and hence this offers a possible functional outcome

(Einstein et al., 2010). In addition to *IGF2/H19* and *IGF2R*, other imprinted genes have been popular candidates because of their key roles in development (Reik et al., 2003) and the fact that they are abundantly expressed in the placenta (Coan et al., 2005). These include *CDKN1C*, *PHLDA2,* delta like homolog 1 *(DLK1),* growth factor receptor-bound protein 10 (*GRB10),* paternally expressed gene 10 *(PEG10)* and zinc finger gene 2 (*ZIM2)* (Tycko & Morison, 2002) (Coan et al., 2005). Non-imprinted genes have been under-represented in studies, in particular *GR* and *HSD11β2* considering their roles in stress regulation. Of additional interest is peroxisome proliferator-activated receptor-gamma (*PPARγ)* which is proposed to have a pivotal role in placental development and adipogenesis and expression has been shown to vary with birth weight (Diaz et al., 2012). Several studies have examined both DNA methylation and its relationship with gene expression. Most prior studies have compared IUGR/SGA (with variable definitions of IUGR and SGA) vs. AGA pregnancies with inclusion of preterm pregnancies with slow intra-uterine growth in the IUGR/SGA cases (Tables 1.1 to 1.4), but as morbidity and mortality is graded across the normal birth weight range with the lightest and heaviest babies being most affected (Basso et al., 2006) it would be important to treat weight for gestation as a continuous rather than a dichotomous variable.

Study	**Tissues**	***N***	**Genomic regions**	**Main findings**
McTernan et al.,2001	Placenta	19 IUGR, 86 AGA across normal gestation	*11βHSD2*	Expression increases as gestation advances, but reduced expression in IUGR
Apostodilou et al., 2007	Placenta	200	*IGF2, MEST, PHLDA2, IGF2R*	Upregulation of *PHLDA2* with decreasing birth weight
Diplas et al., 2009	Placenta	7 IUGR (includes preterm), 10 AGA	74 imprinted genes	Differential expression of 9 genes in IUGR. Upregulated: *PHLDA2, ILK2, NNAT, CCDC86, PEG10*. Downregulated: *PLAGL1, DHCR24, ZNF331, CDKAL1*. No correlation between expression and loss of imprinting.
Börzsönyi et al., 2011	Placenta	101 IUGR (includes preterm), 140 AGA	*IGF1, IGF2, IGFBP3*	Upregulation of *IGF2* and *IGFBP-3 in* IUGR
Kumar et al., 2012	Placenta	39 Preterm (24-36 weeks), 24 term (10 IUGR)	*IGF1, IGF1R, IR, IGF2, IGF2R, GRB10, PHLDA2, H19, PEG1, PEG3, ZNF127*	Upregulation of *PHLDA2* and *IGF2R* in IUGR. *ZNF127, IGF1* and *PHLDA2* varied with gestation
Moore et al., 2015	1st trimester chorionic villi samples (CVS) and term placenta	355 CVS, 302 term placenta	15 imprinted genes	Positive correlation between *IGF2* and *IGF2R* in CVS with birth weight. Negative correlation between *PHLDA2* and *GRB10* in placenta with birth weight and OFC respectively.
Kappil et al., 2015	Placenta	677 term	All 108 established and putative imprinted genes	*BLCAP, DLK1, H19, IGF2, MEG3, MEST, NNAT, NDN* and *PLAGL1* upregulated in LGA. In SGA, upregulation of *MEST* and downregulation of *NNAT*.

Table 1.1 Studies of gene expression in the placenta in relation to birth weight

Study	Tissue	*N*	Genomic region	Main findings
Banister et al., 2011	Placenta (maternal side)	89 IUGR/SGA, 117 AGA	Infinium HumanMethylation27 BeadChip array	5mC profile over 22 genes (5 classes) associated with IUGR/SGA
Filiberto et al., 2011	Placenta	102 SGA, 343 AGA, 35 LGA	*GR* promoter exon 1F	Increased 5mC with LGA
Wilhelm-Benartzi et al., 2012	Placenta	380	*LINE-1* and *AluYb8*	5mC correlated with birth weight
Gordon et al., 2012	Placenta and cord blood	8 monozygotic and 7 dizygotic twin pairs	Infinium HumanMethylation27 BeadChip array	5mC associated with birth weight at genes linked to metabolism, growth and cardiovascular disease in cord blood, but not in the placenta
Hillman et al., 2015	Placenta (fetal side) and cord blood	22 IUGR/SGA, 23 AGA	Infinium HumanMethylation450 BeadChip array	Distinct 5mC profile in cord blood in term IUGR and placenta in preterm IUGR. DMRs in gene regulation, transcription pathways in organ development and metabolism (term), autophagy, oxidative stress and hormones (preterm)

Table 1.2 Studies of DNA methylation in the placenta in relation to birth weight

Study	Tissues	*N*	Genomic regions	Main findings
McMinn et al., 2006	Placenta	38 IUGR, 75 AGA	*PHLDA2* and *MEST*	Increased *PHDLA2/MEST* ratio in IUGR. Not accompanied by changes in 5mC
		14 IUGR, 15 AGA	Affymetrix U133A microarray	Differential expression of imprinted genes, endocrine signalling, tissue growth, immune modulation, oxidative metabolism, vascular growth and solute transport
Guo et al., 2008	Placenta (fetal side)	24 SGA, 20 AGA	11p15.5	Decreased expression of *IGF2* in SGA. Altered 5mC at *H19 ICR* with bi-allelic expression of *H19* in one SGA placenta.
Bourque et al., 2010	Placenta (fetal side)	Discovery group: 22 normal, 13 IUGR, 17 pre-eclampsia, 21 pre-eclampsia and IUGR	GoldenGate Methylation Cancer Panel 1 array	No significant change in 5mC
		Group for candidate genes: 5 normal, 5 IUGR, 4 pre-eclampsia (includes preterm)	*H19 ICR, KvDMR, CDKN1C* promoter, *H19* promoter, *PEG10, PLAG1, SNRPN, MEST, LINE-1*	Decreased 5mC at *H19 ICR* in normotensive IUGR
			HumanRef-8 v2 BeadChip gene expression array	Reduced expression of *IGF2*, but not when verified by qPCR
Tabano et al., 2010	Placenta, umbilical cord and cord blood	66 IUGR, 60 AGA	*IGF2/H19*, miRNA 483-3p (*IGF2* intron 2) and *LINE-1*	No difference in 5mC; 5mC does not relate to gene expression

Table 1.3 Studies of DNA methylation and gene expression in the placenta in relation to birth weight

Study	Tissues	*N*	Genomic regions	Main findings
Koukoura et al., 2011	Placenta	31 IUGR (includes preterm), 17 AGA	*H19*	Increased *H19* expression with decreased 5mC at *H19* promoter in IUGR
Ferreira et al., 2011	Placenta and cord blood	Discovery group: 8 SGA, 8 AGA; Validation group: 170	Antibody enrichment and Human 244 K Agilent CGI microarray	21 differentially methylated regions. Higher 5mC and lower expression of *WNT2* with lower birth weight in placenta
St-Pierre et al., 2012	Placenta (fetal and maternal sides)	50	*IGF2/H19*	5mC at *IGF2 DMR0* and *DMR2* correlated with birth weight and cord blood *IGF2* levels. No association with *IGF2* expression
Turan et al., 2012	Placenta (fetal side) and cord blood	70	GoldenGate methylation, HumanMethylation27 BeadChip and Illumina HumanHT-12 v3 Expression BeadChip arrays	5mC at 23 genes explained up to 87% of the variance in birth weight (far greater than transcriptome profiling), included *GRB10* and shown to correlate with transcriptional control of other genes involved in fetal growth and insulin signalling.
Marsit et al., 2012	Placenta (maternal side)	185	*11βHSD2*	Higher 5mC with lower birth size. Inverse relation between 5mC and gene expression

Table 1.4 Studies of DNA methylation and gene expression in the placenta in relation to birth weight continued

Little is known about the epigenome in preterm infants. Studies so far suggest that DNA methylation changes with respect to gestational age at birth. From an epigenome wide interrogation, DNA methylation in cord blood associated incrementally with gestational age at birth. Implicated were 3 neighbouring genes responsible for organ development – skeletal muscle, brain and haematopoietic system raising the possibility that this may in part confer the pathologies seen in prematurity (Lee et al., 2012). The differences were significant considering the most immature subjects were 30 weeks gestation and the preterm group comprised a minority of the total. These findings were replicated in another genome wide study and additionally at genes important for shaping the epigenome – *DNMTs* and Ten-eleven translocation (*TET1)* (Parets et al., 2013). Changes genome wide were observed with gestation, even at term (Schroeder et al., 2011) and across the trimesters in the placenta which may reflect changing cellular composition, or accumulating environmental and/or stochastic factors (Novakovic et al., 2011). Nevertheless, these are changes seen at birth and may be regarded as risk factors in the aetiology or consequence of the pathologies leading to preterm delivery. Whether any changes persist at term corrected age is not known; though transient alterations at critical periods of development may alter gene expression and possibly organ structure and function with long term effects.

The pathogenesis of cardiovascular disease is thought to be an inflammatory response to constituent risk factors (Libby, 2006). Recent studies have indicated a relationship between global DNA methylation at repetitive genomic regions and degrees of inflammation, obesity and cardiovascular risk (Stenvinkel et al., 2007) (Kim et al., 2010). Women who have had a prior preterm delivery are at increased risk of developing cardiovascular disease, perhaps suggesting a common mechanism (Bonamy et al., 2011), and the observation that very preterm birth appears to have inflammatory antecedents (Goldenberg et al., 2000) raises the possibility that epigenetic processes may be involved along the way. The available evidence is limited: higher DNA methylation at the repetitive elements - long interspersed nuclear element-1 (*LINE-1)* in maternal blood during the first trimester, but not cord

blood, was associated with longer gestation and reduced odds of preterm birth (Burris et al., 2012). Out of 8 candidate imprinted genes, pleomorphic adenoma gene-like 1 (*PLAGL1)* in cord blood was found to be differentially methylated in preterm birth following infection/inflammation (Liu et al., 2013). Regardless of the presence or absence of infection/inflammation, genome wide differences in cord blood are apparent even following preterm labour with intact membranes compared to term controls (Parets et al., 2013).

Pre-eclampsia is a leading obstetric indication for preterm delivery and a cause of IUGR (Goldenberg et al., 2008). Women who develop pre-eclampsia have a higher risk of cardiovascular disease in later life (Bellamy et al., 2007). The commonality for both conditions may be that the women were born preterm or IUGR, as disorders of placental dysfunction tend to occur across the generations and it has been suggested that this may be due to genetic and/or shared environmental factors (Wikström et al., 2011). Indeed, it has been recognised that the risk of hypertension is increased if the subject has a hypertensive parent (Wang et al., 2008) and several studies have identified multiple common genetic variants that predispose to hypertension (Padmanabhan et al., 2015). Likewise, the familial nature of pre-eclampsia and common genetic variants have been recognised. This may explain the association between prematurity and later high blood pressure, but the data from Johansson et al and Lewandowski et al indicate that the degree of prematurity relates to the severity of the hypertension (Johansson et al., 2005) and cardiac structural changes (Lewandowski et al., 2013) suggesting environmental factors play a role. Epigenetic processes are also implicated in pre-eclampsia. For example, altered DNA methylation has been described in early onset pre-eclamptic placentas identified from genome-wide (Chu et al., 2014) and candidate gene approaches (controlling cortisol and hormonal signa1ling) (Hogg et al., 2013). However, it is unknown whether these changes are causal or consequential.

Are early changes in DNA methylation in preterm infants detectable in the long term? An epigenome wide study using DNA extracted from stored neonatal blood spots showed that differences in methylation profiles between preterm and term infants had mostly resolved by 18 years of age (Cruickshank et al., 2013).

Nevertheless, some of these loci showed persistent changes in DNA methylation at both time points suggesting that these may offer biomarkers of risk, although it is not known whether they associate with risk factors for disease (Cruickshank et al., 2013). Decreased DNA methylation at *IGF2 DMR0* was seen in adults born preterm and with cardiovascular risk factors (Wehkalampi et al., 2013) but whether the changes were present in early life is not known. Differential gene expression and DNA methylation (of several genes including *CDKN1C*) in peripheral blood associated with postnatal growth in ex-preterm and term born children with replication in cord blood (Relton et al., 2012) (Groom et al., 2012). Despite all these studies being correlative and not causative, epigenetic profiles may still offer biomarkers of disease risk in preterm infants.

1.3.3.3 5-hydroxymethylcytosine

In 2009, a second DNA modification, 5-hydroxymethylcytosine (5hmC) was re-discovered having originally been described in bacteriophages in 1953 and again in 1972 in animal brain tissue (Wyatt et al., 1953) (Penn et al., 1972). DNA of mouse Purkinje neurons and embryonic stem (ES) cells were found to contain significant levels of 5hmC (Kriaucionis & Heintz, 2009). Also described were the enzymatic oxidation reactions involving the Ten-eleven translocation (TET) proteins that are responsible for converting 5mC to 5hmC (Tahiliani et al., 2009) (Figure 1.1). This is an active and energy consuming process (as opposed to passive loss of 5mC during cell division in the absence of DNMTs). Particularly as this was observed in post-mitotic neurons, the suggestion is that 5hmC may behave as a stable mark with regulatory function in its own right rather than merely an intermediate in the de-methylation pathway (Tahiliani et al., 2009) (Song & He, 2013).Further cytosine modifications, 5-formylcytosine and 5-carboxylcytosine, have been described as oxidative derivatives of 5hmC also involving the action of TET proteins (Ito et al., 2011). Thereafter, it has been found that 5-carboxylcytosine is excised from DNA by base excision repair machinery to revert to unmodified cytosine (He et al., 2011). This additionally suggests that 5hmC can be part of an active demethylation cycle (Maiti & Drohat, 2011).

Increasing evidence points towards 5hmC having a role in chromatin structure and gene regulation. From analyses of human and mouse ES cells and neuronal genomes, 5hmC is enriched in gene bodies (Williams et al., 2011), in greater amounts at those that are more highly expressed (Song et al., 2011) and with correspondingly low enrichment of 5mC (Mellén et al., 2012), indicating that 5hmC can activate and/or maintain gene expression. 5hmC is also present at enhancer elements (Stroud et al., 2011), CpG-rich transcription start sites (Williams et al., 2011), promoters and insulator binding sites (Shen & Zhang, 2013) indicating, again, that 5hmC may have a role in initiating and maintaining transcription. The function of 5hmC at other regions is unknown, where it may indeed be a transient modification (Hahn et al., 2014).

5hmC varies greatly between tissues. It is approximately 10-fold more abundant in neurons than in ES cells or peripheral tissues and its location in the genome also varies between the neurons and ES cells (Szulwach et al., 2011). Given also that neurons are post-mitotic whereas ES cells are proliferative these data suggests that the function of 5hmC depends on tissue type (Mellén et al., 2012) (Nestor et al., 2012). Assuming a model of passive demethylation, 5hmC loss resulting from failure to maintain the mark after DNA replication, one would predict that tissues with a relatively low proliferation rate (e.g. brain) have the highest levels of 5hmC and highly proliferative tissues (e.g. blood or epithelium) would contain the least, as was broadly observed (Nestor et al., 2012).

Whilst the distribution of 5hmC across the genome can differ from 5mC, the two marks often co-exist, and much of this has been gleaned from work that has used techniques specific for 5hmC (Shen & Zhang, 2013). In particular, large areas of 5hmC enrichment have been noted over the *IGF2/H19* locus (Thomson et al., 2013) (Nestor et al., 2012), a region where 5mC has been studied with bisulphite sequencing in the placenta with respect to fetal programming (Tabano et al., 2010) (St-Pierre et al., 2012) (Bourque et al., 2010) (Koukoura et al., 2011) (Buckberry et al., 2012). 5hmC is known to be present in the mature placenta (Nestor et al., 2012), so bisulphite sequencing would not have technically distinguished 5mC from 5hmC (Huang et al., 2010), therefore the role of the latter and less well-studied

modification may have been underestimated. Moreover, the true abundance of 5mC may not have been accurately captured. Yet 5hmC in the placenta has not been studied in the context of fetal programming nor as a potential regulator of placental function.

Finally, since that the action of the TET enzymes requires oxygen, this suggests that it is a process potentially sensitive to environmental states such as oxidative stress and may adapt to altered cellular states (Branco et al., 2011) (Chia et al., 2011). TET mediated conversion of 5mC to 5hmC has been recognised as one mechanism for the global erasure of 5mC, including over imprinted regions, during the development of primordial germ cells during embryogenesis (Hackett et al., 2013). Thus any alterations to this process and also the re-programming that occurs later in gestation may be transmitted to later generations (Rose et al., 2014) and may manifest as aberrant patterns of both 5mC and 5hmC. Therefore, it would be important to study both DNA modifications in the context of fetal programming.

1.3.4 Telomere attrition

Telomeres are strings of highly conserved, repetitive, non-coding DNA sequences that cap the end of chromosomes and preserve genomic DNA integrity (Blackburn, 2005). In the absence of restoration, telomeres shorten during each cycle of mitosis, due to DNA polymerase being unable to replicate the lagging strand completely. Eventually, cells with enough depleted telomeres and essential sequences undergo replicative senescence (Harley et al., 1990) (Blackburn, 2005), thus telomeres are thought of as ‘mitotic clocks’. Telomerase is a ribonucleoprotein that stops the shortening of telomeres by replicating new telomeric DNA in the parent chromosome (Greider & Blackburn, 1985) (Blackburn, 2005). Telomerase is normally expressed in cells where complete replication of DNA (incomplete replication is balanced by replication) is important (such as germ cells and certain adult stem cells) and in cancer cells and levels in somatic cells are low (Kim et al., 1994). The introduction of telomerase into normal cells results in telomere elongation and vigorous cell division (Bodnar et al., 1998). As such, a decline in telomerase activity has also been considered to have a causal role in ageing (Jaskelioff et al., 2011).

Telomere length may be a marker of biological ageing and not just chronological ageing (Lindsey et al., 1991). Although there is an inverse relationship between telomere length and age (Frenck et al., 1998), the wide variation in telomere length at any given age even at birth (Okuda et al., 2002), suggests influence by other factors. There is high heritability of telomere length (Broer et al., 2013) and several genes have been identified that influence telomere length and that are also implicated in age related illnesses such as cancer and coronary artery disease (Codd et al., 2013). This recapitulates the seminal work showing the association between short telomere length and mortality from heart disease (Cawthon et al., 2003). However, the presence of risk factors (insulin resistance and adiposity) alone are linked with telomere attrition (Gardner et al., 2005). Telomeres probably go beyond being biomarkers given the known diseases of telomere dysfunction (dyskeratosis congenita, forms of aplastic anaemia, lung and liver fibrosis) (Calado & Young, 2009) and telomere biology has been linked to mitochondrial function, metabolism and by extension, organ dysfunction and pathogenesis of disease (Sahin et al., 2011).

Environmental factors may also play a major role in modifying telomere length. Lifestyle, nutrition and stress are factors that can predispose to age-related disease and they have been shown to influence telomerase activity in adults (Ornish et al., 2008). A large body of work has focussed on telomere attrition as a means of explaining how stress gets ‘under the skin’ (Epel et al., 2004). Psychological stress in pre-menopausal women has been shown to associate with lower telomerase activity and shorter telomere length (Epel et al., 2004). In childhood, social deprivation associated with shorter telomeres (Mitchell et al., 2014) and exposure to violence associated with telomere attrition (Shalev et al., 2012). Intra-uterine stress (albeit assessed retrospectively) was shown to associate with shorter telomere length in the offspring as adults (Entringer et al., 2011). Given that telomere length in early life is a strong predictor of realised lifespan (Heidinger et al., 2012) and the ‘set-point’ may be established in early life (Daniali et al., 2013), these studies suggest that early adversities can alter the trajectory of attrition with measurable differences in later life. Moreover, the telomere system may be partly under epigenetic control by DNA methylation at subtelomeric loci (Buxton et al., 2014) and the promoter region of the

telomerase reverse transciptase gene (*TERT*) that regulates telomerase activity (Daniel et al., 2012), and folate seems to be a required common substrate (Moores et al., 2011).

Whilst some studies suggest that adults (Entringer et al., 2011) and children (Raqib et al., 2007) born small have shorter telomeres, no differences in telomere length was observed in a large cohort of adults with low birth weight or very preterm birth compared to controls (Kajantie et al., 2012). Of note, this was a cohort where birth status predicted a number of adult diseases and their risk factors such as hypertension, type 2 diabetes or impaired glucose tolerance, use of lipid lowering medication and depressive symptoms (Kajantie et al., 2012).

There are, however, very few studies on telomere biology in early life or in preterm infants. Whilst data do not indicate that fetal growth restriction associates with shorter telomeres at birth (Akkad et al., 2006), it appears that telomere length is inversely proportional to gestational age – at least when measured at a single time point in a range of preterm infants at birth (Friedrich et al., 2001) (Menon et al., 2012). Serial measurements during hospital stay indicated steady shortening of telomeres in preterm infants compared with unborn fetuses matched for gestational age (Holmes et al., 2009) and although this was a very small study (total $n = 13$), it suggests premature cellular ageing in the preterm group. Mechanisms proposed for this include oxidative stress from in vitro studies (Kawanishi & Oikawa, 2004) (Zglinicki, 2002) or inflammation from a human study (Wong et al., 2014). These mechanisms were explored in a mouse model: low grade chronic inflammation induced telomere dysfunction and ageing via reactive oxidative species (Jurk et al., 2014). Both oxidative stress and inflammation are implicated in morbidity in the neonatal period in preterm infants (Vento et al., 2012) (Dammann & Leviton, 2000). These previous studies of telomere biology were limited to the perinatal period, the preterm groups did not have telomere length measured at term corrected age and there was no longitudinal study in infancy or childhood.

As opposed to the mean telomere restriction fragment method used in the study by Holmes et al (Holmes et al., 2009) and Friedrich et al (Friedrich et al., 2001), the

quantitative polymerase chain reaction (qPCR) method (Cawthon, 2002) used by Menon et al (Menon et al., 2012) allows for larger numbers of samples to be analysed. Comparing telomere length measurements of a larger sample of preterm infants with term infants from birth over an equivalent time period is required to determine whether preterm infants have faster telomere attrition and to explore the possibility of telomere length as a marker of early life stress response.

1.4 DNA methylation and preterm brain development

Neurodevelopmental impairment is one of the most important outcomes following preterm birth. Major motor deficits only occur in 5-10% of VLBW infants, but up to 50% can have subtle learning difficulties at school age (Aarnoudse-Moens et al., 2009). There is an inverse relationship between statutory special educational needs (SEN) and gestation at birth up until 41 weeks (MacKay et al., 2010). Here, SEN included behavioural problems such as autistic spectrum disorder (ASD) and ADHD (MacKay et al., 2010). Extreme preterm children are more than 3 times as likely to have such a psychiatric disorder and the presence of inattention, social difficulties and also anxiety comprise a consistent "preterm behavioural phenotype" (Johnson et al., 2010) (Johnson & Marlow, 2011). A Swedish nation-wide study showed that the risk of hospitalisation for psychiatric disorders (most commonly depression, bipolar disorder and psychoses) as adults, rose monotonically with decreasing gestational age (Nosarti et al., 2012) and similarly for prescription of psychiatric medication (Crump et al., 2010).

The main neuropathology in the preterm infant comprising periventricular leucomalacia (PVL) and neuronal/axonal disease, accounts for the large majority of the observed neurodevelopmental abnormalities and collectively, this is now known as "the encephalopathy of prematurity" (Volpe, 2009). PVL consists of focal necrosis deep in cerebral white matter and a more diffuse white matter disturbance. The focal necroses are usually microscopic (macroscopic cysts are now unusual) and the diffuse white matter disturbance is characterised by injury to pre-myelinating oligodendrocytes. The pathology results from a combination of destructive processes and abnormal development (trophic/maturational), with the key insults being

ischaemia, inflammation, excitotoxicity and free-radical injury (Volpe, 2009) (Volpe, 2011).

On conventional MRI, the neuropathology comprises a 'signature' of preterm birth at term equivalent age consisting of enlargement of the ventricular system, reduced cortical complexity and diffuse signal abnormalities in the white matter (Woodward et al., 2006). Advanced MRI tools to capture and analyse images have further delineated how preterm birth affects neural systems (e.g. white matter microstructure (Counsell et al., 2008) and focal volume loss in the deep grey matter and thalami (Boardman et al., 2006) (Boardman et al., 2010)). However, central to the encephalopathy of prematurity are the neuronal/axonal deficits (Volpe, 2009) and diffusion MRI tractography has been able to demonstrate reduced connectivity of neural tracts in preterms at term corrected age in relation to cognitive deficits in toddlerhood (Ball et al., 2015) and also social deficits at school age (Fischi-Gómez et al., 2014).

Diffusion MRI (dMRI) assesses the Brownian motion of water in tissues to define white matter microstructure. Fractional anisotropy (FA), a derived metric, measures the directionality of water diffusion parallel to the fibre tract and detects differences in integrity due to maturation and damage (Le Bihan, 2003). In the preterm brain, the normal increase in FA in various fibre tracts is blunted (Hüppi et al., 1998). This occurs as in the early preterm period the brain is about 92% water (Dobbing & Sands, 1973) and largely unmyelinated (Brody et al., 1987). As the brain matures, cellular and axonal size, density and the coherence of fibres all increase, while the water content declines to 88% at birth. Simultaneously, myelination starts with the development of oligodendrocytes and collectively, there is more restriction in tissue causing increased water directionality (Miller et al., 2002). Thus, FA is a composite measure of all of these factors (Beaulieu, 2002). By studying the preferred direction of diffusion, the orientation of white matter fibre bundles can be inferred and tract averaged FA values can be determined (Basser et al., 2000).

dMRI has demonstrated regions of the brain that are impaired in several brain disorders, for example in a meta-analysis, the splenium of the corpus callosum

showed significantly reduced FA in adults with schizophrenia compared to controls (Patel et al., 2011). dMRI based abnormalities have been shown in other conditions: post-traumatic stress disorder, bipolar mood disorder, obsessive-compulsive disorder and major depression (Daniels et al., 2013) (Sussmann et al., 2009) (Zarei et al., 2011) (Carballedo et al., 2012) but not universally (Kochunov et al., 2013). Studies combining dMRI and genetic variation have increased the understanding of how white matter integrity relates to cognition and the susceptibility to schizophrenia and related disorders (Chiang et al., 2011a) (Chiang et al., 2011b) (Sprooten et al., 2011) (Kohannim et al., 2012) (Carballedo et al., 2012), which are regarded fundamentally as disorders of myelin and connectivity (Davis et al., 2003). Similarly, preterm white matter injury has been related to common genetic variants (from a candidate enquiry) that modulate the risk of schizophrenia and possibly cognition, namely rs2518824 in armadillo repeat gene deleted in velocardiogacial syndrome (*ARVCF)* and rs174576 in fatty acid desaturase 2 (*FADS2)* (Boardman et al., 2014). Common variants in *ARVCF* are also associated with altered FA in adult subjects who have schizophrenia (Sim et al., 2012). *FADS2* encodes for δ-6 desaturase which modifies dietary fatty acids leading to long-chain polyunsaturated fatty acid (LC-PUFA) production that are important for brain development. Most interestingly, the *FADS2* rs174575 genotype alters LC-PUFA availability and carriage appears to interact with breast milk exposure to influence childhood IQ (Caspi et al., 2007).

Common genetic and not epigenetic variation has been the focus of these studies. Since the brain is highly plastic in function, there is constant adaptation in neural function in response to environmental cues; potentially mediated by epigenetic processes, such as DNA methylation (Meaney & Ferguson-Smith, 2010) and DNA methylation is required for proper postnatal brain development (Hutnick et al., 2009). Several studies have indicated the mediation of early life stress and later mental illness by DNA methylation including the control of glucocorticoid action (McGowan et al., 2009) (Essex et al., 2013) (Mehta et al., 2013) (Khulan et al., 2014). As already described, preterm delivery with its associated practices and complications results in abnormal stress regulation and behavioural difficulties. Such early stressors may embed into DNA methylation and underpin acquired brain injury.

In a study of DNA methylation changes in the human pre-frontal cortex across the lifespan, the greatest change occurred during fetal life (Numata et al., 2012) but there is also considerable change during fetal and early postnatal life (Spiers et al., 2015). This suggests that preterm birth may impact greatly on normal methylation during brain development. Whilst recent studies have revealed alterations in peripheral blood or brain tissue in adults with ASD and schizophrenia (Aberg et al., 2014) (Ladd-Acosta et al., 2014) (Pidsley et al., 2014), studies following preterm birth are now emerging (Behnia et al., 2015).

Imprinted genes are recognised to play a key role in normal neurodevelopment and brain function (Wilkinson et al., 2007). For example, children with the well-known imprinting disorders Angelman and Prader-Willi syndromes have cognitive impairment and characteristic behavioural changes and children with Beckwith-Wiedemann syndrome have an increased risk of autism. This had led to the theory that ASD has its origins in aberrant imprinting (Badcock & Crespi, 2006). Similarly, conditions with psychosis (schizophrenia, bipolar mood disorder and depression) are also characterised by disturbance of cognition, affect and behaviour and may too be due to imbalance of imprinting (Badcock & Crespi, 2008). In general, schizophrenia is considered to be a neurodevelopmental disorder and also obstetric complications are an additional risk factor (McGrath et al., 2003) (Cannon et al., 2002). Structural brain abnormalities (focal white matter hyper-intensity and decreased brain volume) (Hulshoff Pol et al., 2000) and altered DNA methylation at *IGF2/H19* locus (Heijmans et al., 2008) have been demonstrated following severe perinatal adversity in the form of maternal undernutrition during the Chinese and Dutch famines (St Clair et al., 2005) (Susser & Lin, 1992). Since *IGF2* is expressed in the brain in a parent of origin specific manner, and has been shown to have roles in brain function such as cognition and memory (Chen et al., 2011) and in synapse formation (Schmeisser et al., 2012), altered DNA methylation at *IGF2/H19* could be one mechanism in preterm brain injury.

1.5 Search for biomarkers of risk

Epigenetic mechanisms and telomere attrition may be potential explanations for the effects of early life environmental effects on developmental trajectories. If the 'signature' is present before the development of the phenotype, it could be used as a biomarker of disease risk (or resilience) rather than relying on the surrogate marker of low gestational age or low birth weight. Identifying those preterm infants who are most at risk of later disease would allow the development and targeting of appropriate therapies.

Appropriate accessible tissue from preterm infants is a source of challenge for genomic studies. It is impractical to study brain tissue. Peripheral white cells may be a more direct means of understanding the role of inflammation on the pathogenesis of both brain injury and cardio-metabolic disease, but collecting blood from infants poses ethical as well as practical hurdles. The composition of peripheral blood can also vary and this would need to be taken into account. Buccal epithelium consists of more homogenous cells and is also easily accessible, but must be seen as a surrogate marker. The placenta is also highly accessible, but offers information only of intra-uterine events. Whilst telomere length may differ between tissues, the rate of attrition is equivalent in epithelia compared with other somatic tissues (Daniali et al., 2013). DNA methylation differences can be tissue specific, but some regions may be conserved (Ollikainen et al., 2010) (Schultz et al., 2015) and research so far has indicated that buccal epithelia may be superior to blood for the study of human disease including brain disorders (Smith et al., 2014) (Lowe et al., 2013). Ultimately, it may be possible to use epigenetic signatures for diagnostic purposes once tissue specific profiles for diseases are known and the most suitable accessible tissue can be chosen.

1.6 Hypotheses

1. Preterm infants have altered DNA methylation at *IGF2/H19* over the first year of life compared with term infants.

2. DNA methylation at the *IGF2/H19* locus relates to fractional anisotropy (FA), a measure of microstructural integrity of key white matter tracts, and/or whole brain volume in preterm infants.

3. Preterm birth is associated with faster telomere attrition during the first year of life compared with term infants.

4. The expression of candidate imprinted and non-imprinted genes that mediate fetal growth in the placenta associate with birth weight.

5. Placental DNA methylation (5-methylcytosine) and 5-hydroxymethylcytosine at candidate genes associate with birth weight.

1.7 Aims

1. Establish a cohort of preterm infants (23 to 31 weeks gestation) and full term controls within the first week of age. Follow up the infants at term corrected age, 3 months and 1 year corrected. Measure growth parameters, including body composition, and collect saliva for buccal epithelial DNA and cortisol. Study DNA methylation at the *IGF2/H19* locus.

2. Establish a pathway for the acquisition of magnetic resonance (MR) images of the brain of preterm infants at the University of Edinburgh, Clinical Research Imaging Centre. Establish recruitment of preterm infants, supervise their scanning at term corrected age, collect saliva for buccal epithelial DNA and study DNA methylation at the *IGF2/H19* locus.

3. Measure relative telomere length at birth, term corrected age and 1 year corrected in the same cohort of infants.

4. Study expression of candidate imprinted and non-imprinted genes in term placental samples that represent the normal birth weight range obtained from the Edinburgh Reproductive Tissue Biobank.

5. Study epigenetic control mechanisms that might affect gene dosage using new affinity based techniques that would distinguish between DNA methylation (5

methylcytosine) and 5-hydroxymethylcytosine allowing exploration of their individual relationship with birth weight.

Chapter 2: Materials and methods

2.1 Clinical methods

2.1.1 Cohort of preterm and full term infants

Following written parental consent, 50 preterm and 40 full term infants were recruited within the first week of age. Recruitment started on 12 July 2011 and ended 11 November 2012. All infants were born at the Royal Infirmary of Edinburgh, apart from one full term infant who was born at St John's Hospital, West Lothian.

The inclusion criteria were preterm infants born at < 32 weeks gestation with no congenital abnormality. For the full term infants, inclusion criteria were: singleton babies born between 37 and 42 weeks gestation of mothers who did not smoke during pregnancy, did not undergo assisted reproduction and had no chronic health condition that would impact fetal growth. This was in line with an existing research study at the University of Edinburgh examining outcomes following birth to women with a body mass index exceeding 40kg/m^2. The inclusion criteria for the term control group matched those of my study. This facilitated identification for recruitment by myself and research midwives and participation in both studies. Excluding women with adverse risk factors tries to ensure that the newborns are as healthy as possible. This is likely to exaggerate differences observed against infants born after an abnormal pregnancy. However, keeping the inclusion criteria strict allows the cohort to be similar as possible to each other and offered a valuable resource for subsequent study of DNA methylation in preterm infants in The University.

For the purposes of obtaining anthropometric measures including body composition and collecting biological samples, infants were seen within the first week of age ("birth"), 3 months/3 months corrected for preterm infants ("3 months") and 1 year/1 year corrected ("1 year"). Additionally, the preterm infants were seen at a postmenstrual age of 37 to 42 weeks ("term corrected age"). All time points mentioned henceforth refer to the corrected age denoted within brackets. Follow up visits were conducted at the Clinical Research Facilities at The Royal Infirmary of Edinburgh and Royal Hospital for Sick Children Edinburgh. All follow up

arrangements were made by myself. All follow up visits were conducted by myself, except in a few exceptional circumstances, and completed by 14 October 2013. Demographic details and history were obtained during follow up visits and from the infant and the mother's hospital records.

For all term infants, mothers were approached in the antenatal period in order to obtain consent for cord blood, maternal blood and placental samples. This was also achieved for 30% of preterm infants. These samples were collected under the Edinburgh Reproductive Tissue BioBank (ERTBB) which works with ethical and governance approval from the Scottish Academic Health Sciences Collaboration, Human Annotated BioResource (East of Scotland Research Ethics Service 13/ES/0126) and previously the West of Scotland Research Ethics Committee (REC) 4 (09/S0704/3). I participated on a rota, both working hours and out-of-hours, for the collection and processing of samples for the ERTBB. Infant samples were collected under the framework of the ERTBB following an amendment (Reference AM07/1, 17 March 2011).

For the purposes of establishing the cohort of infants, substantive ethical approval was obtained from the South East Scotland REC (Reference 11/AL/0329) on 15 Jun 2011. National Health Service (NHS) management approval was obtained on 30 June 2011 (Lothian Research & Development Project number 2011/R/NE/03). These approvals included permission for 15 of the 50 preterm infants to be enrolled into a pilot study of brain imaging using magnetic resonance. At the end of recruitment, these infants had participated in brain imaging under REC Reference 11/SS/0061 with approvals to enrol a larger group of preterm infants into brain imaging (Section 2.1.2). However, in order to collect saliva for DNA analysis from these infants, an amendment (Reference AMO1-1, 30 July 2012) to the initial application (Reference 11/AL/0329) permitted this. The permission was to collect saliva for DNA analysis to study DNA methylation and common genetic variants in relation to brain development from up to a further 100 preterm infants undergoing brain scanning.

Number	Maternal blood	Placenta	Cord blood	Saliva for DNA	PeaPod	3 months	PeaPod	Saliva for cortisol	Saliva for DNA	1 year	Saliva for DNA
1	yes	yes	yes	yes	not done	withdrew from the study					
2	yes	yes	yes	yes	not done	seen	yes	yes	yes	seen	no
3	yes	no	yes	yes	not done	seen	yes	yes	yes	seen	yes
4	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
5	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
6	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
7	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
8	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
9	yes	yes	yes	yes	yes	seen	machine error	yes	yes	seen	yes
10	yes	yes	yes	yes	not done	seen	yes	yes	yes	seen	yes
11	yes	yes	yes	yes	not done	seen	yes	yes	yes	seen	yes
12	yes	yes	yes	yes	yes	not seen				withdrew	
13	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
14	no	yes	yes	yes	yes	not seen				withdrew	
15	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
16	no	yes	yes	yes	not done	seen	yes	yes	yes	seen	yes
17	no	yes	yes	yes	not done	seen	not done	yes	yes	seen	yes
18	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
19	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
20	no	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes

Table 2.1 Term infants

Number	Maternal blood	Placenta	Cord blood	Saliva for DNA	PeaPod	3 months	PeaPod	Saliva for cortisol	Saliva for DNA	1 year	Saliva for DNA
21	yes	yes	yes	yes	not done	seen	yes	yes	yes	withdrew	
22	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
23	no	no	yes	yes	yes	seen	yes	yes	yes	seen	yes
24	no	yes	yes	yes	yes	not seen				seen	yes
25	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
26	no	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
27	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
28	no	yes	yes	yes	yes	seen	not done	no	yes	seen	yes
29	no	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
30	no	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
31	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
32	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
33	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
34	yes	no	yes	yes	yes	seen	yes	yes	yes	seen	yes
35	no	yes	yes	yes	yes	seen	not done	yes	yes	seen	yes
36	yes	yes	yes	yes	yes	not seen				seen	yes
37	no	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
38	yes	yes	yes	yes	yes	seen	yes	yes	yes	seen	yes
39	yes	yes	yes	yes	yes	seen	yes	yes	yes	resident in England	
40	yes	yes	yes	yes	yes	seen	machine error	yes	yes	seen	yes

Table 2.2 Term infants continued

Number	Maternal blood	Placenta	Cord blood	Peripheral blood	Saliva for DNA	Term age	Saliva for DNA	PeaPod	MRI brain	3 months	PeaPod	Saliva for cortisol	Saliva for DNA	1 year	DNA
41	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
42	no	no	no	yes	yes	seen	yes	BPD		not seen				seen	yes
43	no	no	no	yes	yes	seen	yes	central line		seen	central line	no	yes	seen	yes
44	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
45	yes	yes	yes	yes	yes	seen	yes	not done		seen	yes	yes	yes	seen	yes
46	no	no	no	yes	yes	seen	yes	not done		seen	yes	yes	yes	seen	yes
47	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
48	no	yes	yes	yes	no	seen	yes	yes		seen	not done			seen	yes
49	no	no	no	no	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
50	yes	yes	yes	no	yes	died									
51	yes	yes	no	yes	no	seen	yes	BPD		seen	yes	yes	yes		
52	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
53	yes	yes	yes	no	yes	seen	yes	BPD		seen	yes	yes	yes	seen	yes
54	yes	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
55	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	not seen	
56	yes	yes	yes	no	yes	seen	yes	central line		not seen				not seen	
57	yes	no	no	no	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
58	no	no	no	yes	yes	not seen				not seen				not seen	
59	no	no	no	yes	no	seen	yes	BPD		not seen				seen	yes
60	yes	no	no	yes	yes	not seen				seen	yes	yes	yes	seen	yes

Table 2.3 Preterm infants

Number	Maternal blood	Placenta	Cord blood	Peripheral blood	Saliva for DNA	Term age	Saliva for DNA	PeaPod	MRI brain	3 months	PeaPod	Saliva for cortisol	Saliva for DNA	1 year	DNA
61	yes	no	no	yes	no	not seen				seen	yes	yes	yes	seen	yes
62	yes	yes	yes	yes	no	died									
63	yes	yes	yes	yes	no	died									
64	no	no	no	yes	no	seen	no	died							
65	no	no	no	yes	yes	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
66	no	no	no	yes	no	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
67	no	no	no	yes	yes	seen	yes	central line		not seen				not seen	
68	no	no	no	yes	no	died									
69	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
70	no	no	no	yes	no	seen	yes	yes	yes	seen	cancelled	yes	yes	seen	yes
71	no	no	no	yes	no	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
72	no	no	no	yes	no	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
73	no	no	no	yes	no	seen	yes	BPD		seen	yes	yes	yes	seen	yes
74	no	no	no	no	no	seen	yes	central line		not seen				withdrew	
75	yes	yes	yes	no	no	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
76	yes	yes	yes	no	no	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
77	yes	yes	no	yes	yes	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
78	yes	yes	yes	no	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes
79	no	no	yes	no	yes	seen	yes	BPD	yes	not seen				seen	yes

Table 2.4 Preterm infants continued

Number	Maternal blood	Placenta	Cord blood	Peripheral blood	Saliva for DNA	Term age	Saliva for DNA	PeaPod	MRI brain	3 months	PeaPod	Saliva for cortisol	Saliva for DNA	1 year	DNA
80	no	no	no	yes	yes	seen	yes	BPD	yes	seen	yes	yes	yes	seen	yes
81	no	no	no	no	yes	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
82	no	no	no	no	no	seen	yes	not done	yes	seen	not done	yes	yes	seen	yes
83	no	no	yes	no	yes	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
84	yes	yes	yes	no	yes	not seen				not seen				seen	yes
85	no	no	no	no	no	seen	*yes*	yes	yes	seen	yes	yes	yes	seen	yes
86	yes	yes	yes	no	yes	died									
87	yes	yes	yes	no	yes	seen	yes	BPD		seen	yes	yes	yes	seen	yes
88	no	no	no	yes	yes	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
89	yes	yes	yes	no	yes	seen	yes	yes	yes	seen	yes	yes	yes	seen	yes
90	no	no	no	yes	yes	seen	yes	yes		seen	yes	yes	yes	seen	yes

Table 2.5 Preterm infants continued

Term and preterm infants described here were recruited under REC Reference 11/AL/0329. Preterm infants that participated in brain imaging was under REC reference Reference 11/SS/0061.

2.1.2 Establishing a pathway for brain imaging of preterm infants using magnetic resonance

The objective was to establish a pathway for brain imaging in preterm infants in Edinburgh and to obtain high-resolution magnetic resonance images at 3 Tesla that would be suitable for understanding brain growth during early development. Eligible babies were preterm infants born at < 32 weeks gestation, with no major congenital abnormality, cystic periventricular leucomalacia, post-haemorrhagic ventricular dilatation or porencephalic cysts. Brain imaging was obtained at 38 to 42 weeks corrected age.

My tasks included compiling a study protocol, obtaining ethical and NHS management approval and obtaining approval from the Clinical Research Facility, which manages the Clinical Research Imaging Centre. Obtaining the necessary approvals required liaising with imaging scientists, radiographers, research nurses, pharmacists and personnel in medical physics to tackle all practical aspects of the study. We obtained approval to sedate infants with chloral hydrate, and although ultimately no infant was sedated, initial preparations involved an added layer of complexity. I explored arranging training in neonatal resuscitation for the research nurses and radiographers and collated materials and equipment from the neonatal unit and medical physics for the conduct of the study and for use in the case of an emergency. I created a checklist for setting up the resuscitaire and ensuring adequacy of equipment prior to each session. I also created the documentation to be completed by medical and nursing staff for each infant, noting relevant history, physical examination and observations of vital signs confirming that the infant is fit for the scan and afterwards, fit for discharge home. I also created the documentation to gather all the relevant clinical data from clinical records and history from parents and the electronic databases that have continued to be in use.

The South East Scotland REC granted approval to enroll 50 preterm infants (Reference 11/SS/0061) on 8 November 2011 and NHS management approval was obtained (Lothian Research & Development Project number 2011/R/NE/04) on 22 December 2011. I enrolled and supervised the scanning of the first 18 infants, starting on 20 April 2012, collected clinical data and samples for DNA and ensured

smooth running. Thereafter the process was taken over by Dr James Boardman's students - Drs Sarah Sparrow, Rozalia Pataky and Emma Moore – with the retention of samples for DNA for my study. Data from infants scanned up until 14 October 2013 was used for this thesis.

2.1.3 Clinical assessments

2.1.3.1 Anthropometry

Birth weight was extracted from the patient record. Thereafter, weight was measured using electronic scales (Seca, Birmingham, UK) to the nearest 0.05kg. Body length was measured to the nearest 0.5cm with a measuring mat (Seca, Birmingham, UK) in full extension using two people. OFC was measured to the nearest mm with a non-stretch tape (Spentex BCA Ltd, Sherburn in Elmet, UK) and the largest measurement of three recorded.

2.1.3.2 PEAPOD

Percentage body fat mass was measured using the PEAPOD Infant Body Composition System (COSMED, Chicago, USA) after birth in the full term infants, at term corrected age in the preterm infants and 3 months in both groups. The weight limit for the PEAPOD is 8kg therefore it was not used beyond 3 months of age.

The PEAPOD uses densitometry to determine body composition and has been validated against the gold standard 4 compartment model and deuterium dilution (Ma et al., 2004) (Ellis et al., 2007).

The infant was undressed and weighed using the integral scale to the nearest 0.1g. Any items on the infants that could not be removed such as umbilical clamps, identification bracelets, and in one instance, a naso-jejunal tube, were used to tare the scale and volume chamber. Following mass measurement, the infant was placed in the chamber of the PEAPOD for 2 minutes and body volume was measured using air displacement plethysmography. The PEAPOD calculated body density by dividing the mass by the volume. Body mass was modeled into two compartments: fat mass and fat free mass. The density of fat is constant throughout life (0.9007 g/ml), and the density of fat free mass was assigned by the PEAPOD depending on the infant's

age and gender and based on published data. The PEAPOD's model also takes into account changes in total body water in the first 6 days post-partum from published data. From this, fat mass and fat free mass were calculated and expressed as percentage of body mass. The Fomon equation was used as it has been shown to be better than that of Butte (Eriksson et al., 2011).

The PEAPOD was stationed in the Clinical Research Facility at the Royal Infirmary of Edinburgh and was calibrated prior to each use. A cylinder with a known volume and a 2kg weight, both supplied by COSMED, were used to calibrate the chamber and the scale.

2.1.3.3 Skin fold thickness

Skin fold thickness (SFT) was measured at 1 year. The triceps and subscapular regions were more reproducible and more acceptable to the child than biceps and supra-iliac and therefore used for analysis. All measurements were made on the left side of the body and using a standard skinfold caliper (Holtain Ltd, Crymych, UK). Triceps SFT was measured parallel to the long axis of the arm midway between the acromion and the olecranon, with the arm slightly flexed. The subscapular SFT was measured below the inferior angle of the scapula at a diagonal in the natural cleavage of the skin. Triplicate measurements were performed and the mean was used.

2.1.3.4 Magnetic resonance image acquisition

Infants were scanned without sedation and whilst asleep, with pulse oximetry, electrocardiography and temperature monitoring. Ear protection was with earplugs and earmuffs (MiniMuffs, Natus Medical Inc, CA, USA).

A Siemens Magnetom Verio 3 T MRI clinical scanner (Siemens AG, Healthcare Sector, Erlangen, Germany) and 12-channel phased-array head coil were used to acquire: T1-weighted magnetisation-prepared rapid gradient echo (MPRAGE) volume scan (~1 mm^3 resolution), T2-weighted short time inversion recovery (STIR), T2-weighted fluid-attenuated inverted recovery (FLAIR), and diffusion MRI (11 T2- and 64 diffusion encoding direction (b=750 s/mm^2) single-shot spin-echo echo planar imaging (EPI) volumes with 2 mm isotropic voxels.

2.1.3.5 MR Image analysis

White matter tract integrity was measured by probabilistic neighbourhood tractography and performed by Dr Devasuda Anblagan. Diffusion MR images were pre-processed using FSL (FMRIB Software Library) (Functional Magnetic Resonance Imaging of the Brain, Oxford, UK; http://www.fmrib.ox.ac.uk) to extract brain, and bulk infant's motion and eddy current induced artefacts were removed by registering the diffusion-weighted to the T2-weighted EPI volume for each subject. Using "DTIFIT", FA volumes were generated for every subject. The BedpostX/ProbTrackX algorithm was run with its default parameters of a 2-fibre model per voxel, 5000 probabilistic streamlines for each tract with a fixed separation distance of 0.5 mm between successive points to generate the underlying white matter connectivity data (Behrens et al., 2003). The tracts of interest (TOI) segmented were: genu and splenium of corpus callosum, left and right projections of the corticospinal tract, cingulum cingulate gyri and inferior longitudinal fasciculi. Reference tracts for these structures were generated from the tractography output produced from a training set of 20 term controls (Anblagan et al., 2015).

Whole brain volumes were computed by Dr Ahmed Serag according to published methods (Serag et al., 2012) Briefly, a 4 dimensional detailed atlas (model) of the brain had been created using 203 preterm infants scanned between 26.7 to 44.3 weeks post menstrual age (PMA). Adjusting for sampling variation at each PMA and change in brain volume over time, the average of the inter-subject variation at each PMA was computed. The resulting probability maps encode the variation in shape of anatomical structures occurring longitudinally and between subjects. This provides prior information on whole brain volumes and also sub-structures. For each infant in the current cohort, registering the images against the reference atlas allowed for computing the anatomical differences between the subject and the atlas and by extension, brain volume. Image analysis was performed blinded to the gestation of the subject.

2.1.4 Sample collection and storage

2.1.4.1 Saliva for cortisol

Saliva was collected using sorbettes (BD Visitec, Massachusetts, USA) and latterly Salimetrics Infant's Swab (Salimetrics Europe, Suffolk. UK) and placed in a storage tube (Salimetrics Europe). Samples were centrifuged at 200 revolutions per minute (rpm) at 4°C for 10 min as soon as possible and stored at -20°C until analysis.

2.1.4.2 Saliva for buccal DNA

The Oragene OG-250 kits with 5 saliva sponges CS-1 (DNA Gentotek, Ottawa, Canada) were used. Saliva was collected at least 30 min after feeding. The solution in the kit stabilises the sample at room temperature and inhibits bacterial growth.

2.1.4.3 Placenta

The samples were collected once the attending midwife had examined the placenta and according to a standard operating procedure. Tissue was excised from the fetal surface, near the insertion of the cord but away from large blood vessels. A $1cm^3$ portion was snap frozen to -80ºC. A second $1cm^3$ portion was placed in RNA*later* Stabilising Reagent (Qiagen, Crawley, UK), refrigerated at 4ºC for 24 hours and then removed from RNA*later* and frozen at -80ºC.

2.2 Laboratory methods

2.2.1 Buffers and solutions

All chemical and reagents were from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

2.2.1.1 10x (immunoprecipitation) IP buffer

100m Molar (M) sodium phosphate pH 7.0 (mono and dibasic), 1.4M sodium chloride and 0.5% Triton X-100 in sterile water

2.2.1.2 10x TBE buffer

890mM Tris base, 890mM boric acid and 20mM (Edetic acid) EDTA pH 8.0 in Milli-Q water (Merck Millipore, MA, USA). Autoclaved and diluted to 0.5x with Milli-Q water before use

2.2.1.3 Phosphate buffered saline – bovine serum albumin (PBS-BSA) 0.1%

A sufficient quantity was constituted prior to use. 1ml bovine serum albumin (10mg/ml stock solution in sterile water, filtered via a 0.22 micron filter and frozen in aliquots) added to 9ml phosphate buffered saline (Oxoid Ltd, Basingstoke, UK) (1 tablet dissolved in 100ml of Milli-Q water and autoclaved).

2.2.1.4 Proteinase K digestion buffer

50mM Tris pH 8.0, 10mM EDTA pH 8.0 and 0.5% SDS in sterile water. A sufficient quantity constituted and filtered via a 0.22 micron filter prior to use.

2.2.1.5 Sodium dodecyl sulphate (SDS) 10%

10g dissolved in 100ml sterile water.

2.2.1.6 Sodium Phosphate buffer

2M sodium phosphate monobasic and 2M sodium phosphate dibasic solutions made and combined (in a ratio of 21:54) to achieve a 1M solution of pH 7.0.

2.2.1.7 TE (Tris EDTA) pH 8.0

10mM Tris pH 8.0 and 1mM EDTA pH 8.0 in Milli-Q water and autoclaved

2.2.1.8 1M Tris pH 8.0

121.14g Trizma base dissolved in 800ml of Milli-Q water. pH adjusted to 8.0 with HCl, made up to 1000ml with Milli-Q water and autoclaved.

2.2.1.9 0.5M EDTA pH 8.0

93.06g EDTA dissolved in 400ml of Milli-Q water. pH adjusted to 8.0 with Sodium hydroxide, made up to 500ml with Milli-Q water and autoclaved.

2.2.2 Salivary cortisol assay

The High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit was used (Salimetrics Europe, Suffolk, UK) and the manufacturer's instructions were followed.

All reagents and the microtitre plate (coated with monoclonal antibodies to cortisol) were brought to room temperature. 25µl of samples and the supplied Controls and

Standards were loaded in duplicate into the appropriate wells of the plate. 25µl of Assay diluent were loaded into 2 wells to serve as a zero value and into 2 wells designated as Non-Specific Binding. 200µl of diluted Conjugate Solution (1:1600 dilution made using Assay Diluent) was added into each well. The plate was mixed on an IKA MS 3 shaker (IKA -Werke, Staufen, Germany) at 500rpm for 5 min and then incubated at room temperature for 55 min. The cortisol in the standards and the samples compete with cortisol linked to horseradish peroxidase for the antibody binding sites. To remove unbound components, the plate was washed 4 times on a Labtech LT-3000 plate washer (Labtech International, East Sussex, UK) with 1X Wash Buffer (Wash Buffer Concentrate diluted 10-fold with distilled water). The plate was then blotted on paper towels. 200µl of tetramethylbenzidine (TMB) solution was added to each well, the plate mixed on the shaker at 500rpm for 5 min and incubated in the dark at room temperature for 25 min. The peroxidase reacts with the substrate TMB and produces a blue colour. The reaction was stopped with 50µl of Stop Solution (sulphuric acid) and a yellow colour was produced. The plate was mixed again on the shaker at 500rpm for 3 min before optical density was read on a plate reader (Molecular Devices OptiMax, Sunnyvale, CA) at 450nm. The minimal concentration of cortisol that can be distinguished from 0 is 0.007µg/dl. The intra-assay coefficient of variation was 10.8%, within the range quoted in the manual for the measured concentrations.

2.2.3 DNA extraction from saliva

The Gentra Puregene Buccal Cell Kit with buccal brushes (Qiagen, Crawley, UK) was tested against the Oragene OG-250 kits with saliva sponges CS-1 (DNA Genotek, Ontario, Canada). The Oragene kits were chosen as they yielded more DNA. However, the manufacturer's protocol does not accommodate the use of RNase when extracting DNA. There is an abundance of RNA in buccal cells and removing it would necessitate re-extracting the DNA and treating with RNase. Potentially, DNA can be lost during the second step therefore we used an alternative method.

First, the sample needed to be recovered from the saliva sponges and the manufacturer's guidance was followed. The sample in the kit was mixed by inversion 5 times and then incubated at 50°C in a hybridisation oven (Techne Hybridiser HB-1D, Bibby Scientific Ltd, Staffordshire, UK) for 2 hours to inactivate nucleases. As much of the free liquid as possible was transferred in to a 15ml conical centrifuge (Falcon) (BD, Oxford, UK) tube. The barrel (without the plunger) of a 5ml sterile disposable syringe (BD) was placed in the same Falcon tube and the sponges from the Oragene kit were transferred into the syringe barrel using sterile disposable forceps (Griprite Blue, Rocialle, Wales, UK). The tube with the syringe barrel containing sponges was centrifuged at 1000 rpm at 20°C for 10 min.

Thereafter, DNA was extracted from the liquid in the tube following instructions in the "Laboratory Protocol for Manual Purification of DNA from whole sample". The volume of the liquid was noted and 1/25th volume of prepIT-2LP reagent (DNA Genotek, Ottawa, Canada) was added, mixed by vortexing for a few seconds and incubated on ice for 10 min to precipitate impurities. The tube was then centrifuged at 20°C for 20 min at 4000 rpm (Eppendorf Centrifuge 5810R, (Eppendorf, Stevenage, UK). The supernatant was transferred to a new 15ml Falcon tube without disturbing the pellet. To precipitate the DNA, 1.2 times the volume of room temperature 100% ethanol was added, mixed by inversion 10 times and then the sample was allowed to stand at room temperature for 10 min to allow the DNA to fully precipitate. The tube was centrifuged at 4000 rpm at 20°C for 10 min and the supernatant was removed as completely as possible. The pellet was washed with 1ml of 70% ethanol by first allowing to stand at room temperature for 1 min, then swirling the tube before removing the ethanol as much as possible. The DNA was rehydrated in 200µl of TE pH 8.0 by first vortexing (Rotamixer, Hook&Tucker Instruments, Croydon, UK) the sample for 30 s and then placing on an orbital shaker (Voss Instruments, Maldon, UK) at room temperature overnight. DNA was stored at 4°C.

2.2.3.1 DNA quality assessment

Quality of DNA was assessed using electrophoresis. 5µl of 100 base pair (bp) DNA ladder (1µg/µl) (Invitrogen, Life Technologies, Paisley, UK) and 2µl of each DNA

sample was mixed with 2µl of Orange-G dye with glycerol and loaded on to an agarose (SeaKem, Lonza, USA) gel (1% in 0.5x TBE buffer) containing nucleic acid gel dye (GelRed 10,000x in water, Biotium, Hayward, CA, USA). Gels were electrophoresed at 100 volts for ~1 h in a Power Pack 200 system (Bio-Rad Laboratories, CA, USA). Gels were visualised with a transilluminator (UVitec, Cambridge, UK) and DNA integrity was determined by the presence of a discrete high molecular weight band and no smearing.

2.2.3.2 DNA quantification

To quantify only the DNA fraction for the downstream applications and to maximise accuracy, we acquired a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Paisley, UK). This technique utilises fluorescence-based dyes that bind specifically to DNA and thus accurately measures the concentration of DNA, whereas a NanoDrop spectrophotometer, which quantifies DNA by absorbance, does not discriminate between DNA and RNA in the same sample.

The Qubit assay was carried out according to the manufacturer's instructions. All Qubit reagents were first brought to room temperature. A 'Working Solution' was prepared by diluting the selected Qubit Reagent 1:200 in Qubit Buffer. The two Standards provided in the kit were assayed each time a Working Solution was prepared and prior to assaying the samples. 10µl of the Standard was added to the 190µl of the Working Solution. 2µl of each sample was added to 198µl of the Working Solution. All tubes were vortexed for 2 – 3 s and incubated for 2 min at room temperature prior to being measured in the Fluorometer.

The ds DNA HS (high sensitivity) reagent was used at first. If the measured concentration exceeded the capacity of the reagent, then the sample was re-measured using ds DNA BR (broad range).

2.2.4 Pyrosequencing

Pyrosequencing of bisulphite converted DNA utilises the sequencing by synthesis principle to measure the percentage methylation at individual cytosines in a given sequence.

2.2.4.1 Bisulphite treatment

Bisulphite treatment deaminated unmethylated cytosines to uracil, whilst methylated cytosines remain unaffected. 500 ng of buccal DNA were treated using the EZ DNA Methylation Gold Kit (Zymo Research Corporation, CA, USA) following the manufacturer's instructions.

First, 900µl of PCR grade water, 300µl of M-Dilution Buffer and 50µl of M-Dissolving Buffer was added to a tube of CT Conversion Reagent and mixed on an orbital shaker (Voss Instruments, Maldon, UK) for 10 min. 130µl of this solution was added to 500 ng of DNA (made up 20µl with PCR grade water). Denaturing of DNA and CT conversion was achieved in a thermal cycler (G-Storm1, Labtech International, East Sussex, UK) with the following steps: 98°C for 10 min, 64°C for 2.5 h and then cooled to 4°C. The sample was loaded into a Zymo-Spin IC Column containing 600µl M-Binding Buffer. After mixed by inverting, the column was centrifuged at full speed for 30 s and the flow through discarded. 100µl of M-Wash Buffer (prepared from concentrate with 100% ethanol) was added to the column and centrifuged at full speed for 30 s. 200µl of M-Desulphonation Buffer added to the column, allowed to stand at room temperature for 15 min and centrifuged at full speed for 30 s. 200µl of M-Wash Buffer was added to the column, centrifuged at full speed for 30 s and the wash repeated. To elute the DNA, 10µl of M-Elution Buffer was added directly to the column matrix placed in a 1.5ml tube and centrifuged at full speed for 30 s. The eluate was diluted with 30µl PCR grade water and stored at -20°C until analysed.

2.2.4.2 PCR amplification of region of interest

Published assays for regions that are known to control the expression of *IGF2* (*DMR2* and the *H19 ICR*) were used. All primers were purchased from Invitrogen (Life Technologies, Paisley, UK) and detailed in Table 2.6. The primers were re-constituted to make a 100µM stock solution. An aliquot was made combining the reverse and the forward primers and diluted with PCR grade water such that the concentration of each primer in this aliquot was 5µM.

1µl of the diluted bisulphite converted DNA was amplified by PCR using 10µl Amplitaq Gold 360 Master Mix (Applied Biosystems, Life Technologies, Paisley, UK), 0.8µl of the forward and reverse primer mix and PCR grade water to make up to 20 to 30µl. The conditions in the thermal cycler were: pre-incubation (95ºC for 10 min), 45 amplification cycles (95ºC for 20 s, the appropriate annealing temperature (Table 2.1) for 20 s and 72ºC for 20 s for elongation). The profile finished with a final extension of 72ºC for 7 min and then cooled to 4ºC.

Randomly selected PCR products and all non-template controls were visualised on a 2% agarose gel along with a 100bp DNA ladder to ensure a discrete band at the appropriate molecular weight, no PCR products in the non-template control and no primer-dimers. Prior to the definitive procedure, this process was performed to ascertain the optimal annealing temperatures.

2.2.4.3 Measuring percentage methylation

The PyroMark Q24 system (Qiagen, Crawley, UK) was used, which utilises a vacuum filtration sample transfer device and Pyrosequencing technology (Biotage AB, Uppsala, Sweden).

10 or 20µl of PCR product was immobilised to 2µl of Streptavidin Sepharose beads (GE Healthcare, Sweden) with 40µl of Binding Buffer (Qiagen, Crawley, UK), and 28 or 18µl of Milli-Q water. The mixture was agitated on an IKA MS 3 Basic shaker (IKA – Werke, Staufan, Germany) for 5 to 10 min at 1400 rpm. The immobilised PCR product was captured and denatured on the PyroMark Q24 Vacuum Prep workstation. The DNA strands were released onto a PyroMark Q24 Plate that had been loaded with 0.75µl of sequencing primer (used at 10µM) mixed with 24.25µl Annealing Buffer (Qiagen, Crawley, UK). After annealing by heating the Plate for 2 min to 80°C using the thermal cycler, the Plate was allowed to cool to room temperature for at least 5 min. The Plate was analysed on a PyroMark Q24 Instrument. A PyroMark Q24 Cartridge was loaded with PyroMark Gold 24 reagents (substrate and enzymes) and nucleotides as stipulated by the PyroMark Q24 Software 2.0. Data were analysed using PyroMark Q24 Software 2.0. Due to the large number of samples, Pyrosequencing was not done in duplicate. Pyrosequencing results that

did not work or were of poor quality as indicated by the Software was removed prior to assignment to group and statistical analysis. Hence, data reported in experimental chapters are less than DNA samples acquired. Dr Jessy Cartier assisted with bisulphite conversion, PCR and Pyrosequencing.

Primer name	Primer sequences	Chromosome location[3]	Annealing temperature	Amplicon size
H19 ICR[1]	F: GGTATTTGGTTTGGGTGATTTA R: ACCCCTCCCACAAAATCTCTAA Seq: TTTGGTTTGGGTGATT	chr11:1979936-1980168	55°C	233bp
IGF2 DMR2 Product 1[2]	F: GGGTTTTGGGTGGGTAGAGT R: CCAAAACAACTTCCCCAAAT Seq: GTTTGGTTTTTTTGAA	chr11:2110839-2111026	60°C	195bp

Table 2.6 Primer sequences for Pyrosequencing

Sequences obtained from 1) Lim et al (Lim et al., 2012) and 2) Dupont et al (Dupont et al., 2004). 3) UCSC Genome Browser Hg 18 assembly. Abbreviations: F - forward; R – reverse; Seq – sequencing. The reverse primers were biotinylated.

Primer name	Primer sequences	Amplicon size
tel 1b tel 2b	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	>76bp
*36B4*F *36B4*R	CAGCAAGTGGGAAGGTGTAATCC CCCATTCTATCATCAACGGGTACAA	75bp

Table 2.7 Primer sequences for the relative telomere length assay

Sequences were obtained from O'Callaghan & Fenech (O'Callaghan & Fenech, 2011)

2.2.5 Relative telomere length assay

Telomere length values were measured from buccal DNA using a validated qPCR assay that determines the relative ratio of telomere repeat (T) copy number to single-copy gene (S) number (T/S ratio) in experimental samples as compared with a reference DNA sample (Cawthon, 2002).

Published primers that were designed to amplify the telomere sequences were used (Table 2.7). 36B4, which encodes the acidic ribosomal phosphoprotein PO located on chromosome 12, was used as the single-copy control gene. The resulting PCR signal is a measure of telomere length, as the number of telomere primers that can bind the telomeric DNA at the beginning of the PCR is directly proportional to the total summed length of all the telomeres in the cell. T/S = 1 when the unknown DNA is identical to the reference DNA in its ratio of telomere repeat copy number to single copy gene copy number.

Relative telomere length was quantified using a LightCycler 480 system (Roche, Burgess Hill, UK). All DNA samples were diluted to 0.4ng/µl concentration with nuclease free water and kept at -20°C until assayed. Pooled buccal DNA from 4 infants from the study sample but collected at age of 6 months was used to create standards. The standards were 4 serial dilutions of 1:10 starting at 10ng/µl. The same standards were used for all PCR runs. Two master mixes of PCR reagents were prepared, one with the T primer pair, the other with the S primer pair. The composition of the 10µl final reaction mix for the T and the S PCRs were identical except for the primers and they contained the following: 1.2ng DNA, 5µl LightCycler 480 SYBR Green I Master, 0.9µl forward primer, 0.9µl reverse primer and 0.2µl LightCycler Water. All primers were purified by High Performance Liquid Chromatography (HPLC) at source and used at a final concentration of 300µM. Telomere PCR and single copy gene PCR assays were done on separate plates whilst preserving the sample grid positions between plates. All DNA samples and standards were run in triplicate. The reaction conditions were: pre-incubation (95ºC for 10 min), 50 amplification cycles (95ºC for 15 s for denaturing and single acquisition at

62ºC for 1 min), followed by a melt curve and then cooling (40ºC for 10 s). Mean concentrations of the PCR products were calculated according to the standard curve method for both T and S separately. Finally, the telomere length (TL) relative to the amount of single copy transcript was calculated using the ratio T/S.

2.2.6 RNA extraction from placenta

Tissue homogenisation and RNA purification and was carried out using RNeasy Fibrous tissue Mini Kits (Qiagen, Crawley, UK) according to the manufacturer's instructions (November 2006 edition).

2.2.6.1 Homogenisation

The RNeasy spin columns can be used to process a maximum of 30mg of tissue but as the tissue in RNA*later* Stabilising Reagent is dehydrated it was important to weigh the tissue and I aimed for between 15 to 20mg of tissue. The remainder of the procedure was done at room temperature. Tissue was placed in round-bottomed 2ml microtubes with 300µl Buffer RLT plus 10µl/ml of β-mercaptoethanol and one 5mm stainless steel bead (Qiagen, Crawley, UK). Thereafter, tissue was immediately homogenised using the TissueLyser (Qiagen, Crawley, UK) at 25Hz for 2 min. The tubes were re-arranged so that the outermost tubes are innermost and vice versa and the homogenisation repeated.

2.2.6.2 Total RNA purification

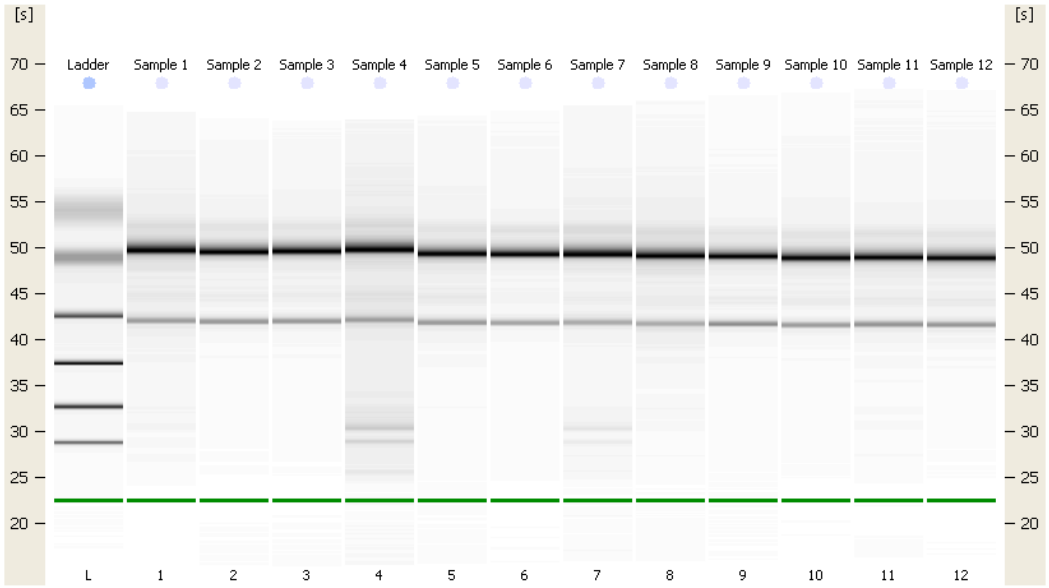
All centrifugation was done at 10,000 rpm unless stated otherwise. The lysate was transferred into a new tube after short centrifuge. 590µl nuclease free water plus 10µl proteinase K added, mixed by pipetting and incubated at 55ºC for 10 min on a heat block. The lysate was centrifuged for 3 min. The supernatant was transferred to a new tube and 450µl 100% ethanol added and mixed by pipetting. 700µl of this solution was placed in a spin column in 2ml collection tube and centrifuged for 15 s. The eluate was discarded and the process repeated with the remainder of the lysate. The spin column was washed with 350µl of Buffer RW1 by centrifuging for 15 s and the eluate discarded. 80µl of DNase I (made up of 10µl DNase I stock solution to 80µl Buffer RDD) was added directly to the spin column and incubated for 15 min. The spin column was washed with 350µl of Buffer RW1 followed by 500µl of

Buffer RPE, centrifuged for 15 s and the eluate discarded after each spin. A final wash was with 500µl Buffer RPE and centrifuged for 2 min. The spin column were placed in clean 2ml collection tubes and centrifuged at full speed for 1 min to dry the membrane and eliminate any possible carryover of the buffer. To elute the RNA, the spin columns were placed in new collection tubes and 30µl of nuclease free water was added directly to the membrane and centrifuged for 1 min. This step was repeated using the eluate to obtain high concentration of RNA. RNA was immediately placed on ice for the purposes of quantification and quality assessment and thereafter stored at -80ºC.

2.2.6.3 RNA quality assessment

A 2100 Bioanalyser system with the RNA 6000 Nano kit (Agilent Technologies, Cheshire, UK) was used and the manufacturer's instructions (April 2007 edition) were followed. The RNA 6000 Nano microchip contains a sieving polymer and a fluorescent dye. When samples and ladder are loaded into the wells, the chip becomes an electrical circuit. As in normal gel electrophoresis, the RNA is driven by the voltage gradient and the molecules separated by size. The dye molecules integrate into the RNA and the speed of movement can be compared to a reference ladder. The provided software translates the data into a virtual 'gel' image and electropherograms. Samples were regarded to be of good quality if two discrete bands on the 'gel' image and on the electropherogram, two peaks were shown corresponding to 28S and 18S RNA. Degradation products, if present, will be evident on both the gel image and the electropherogram. Sample 4 in Figure 2.1 (a) and (b) shows an example of degradation products. For most samples, a RNA Integrity Number (RIN) was available. An algorithm that analyses the entire electrophoretic trace generates the RIN and a score of 1 to 10 is assigned. A sample with a RIN of 8 or above was considered suitable for further analysis. Sample 4 in this example had a RIN of 8.1 and was included in downstream analyses.

a)



b)

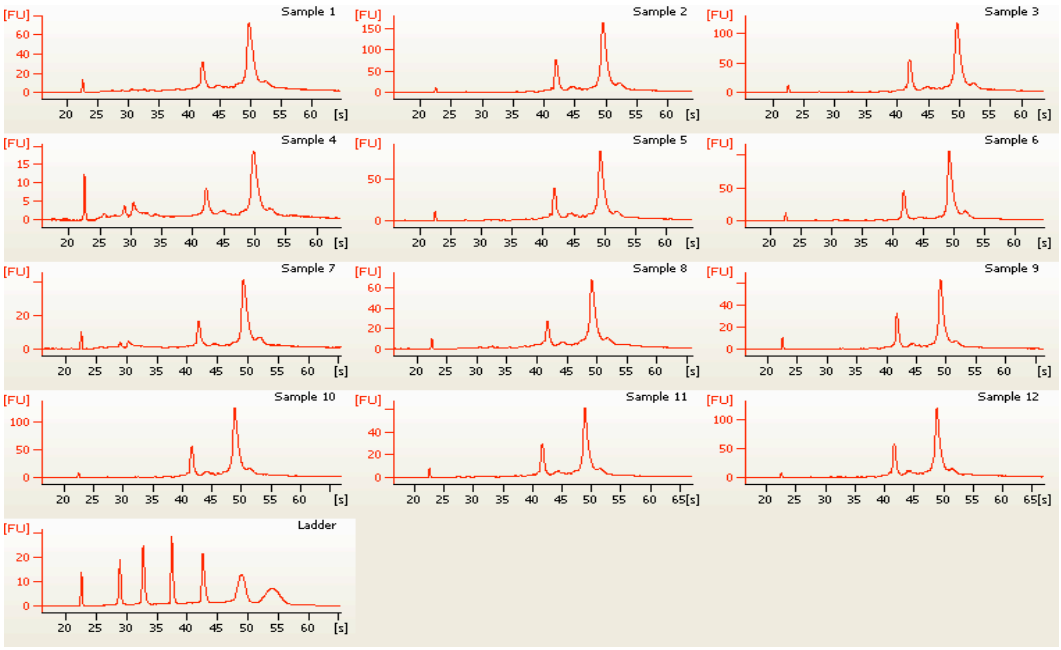


Figure 2.1 Representative Bioananalyzer 'gel' and electropherogram

The dark and light bands on the gel (a) correspond to 28S and 18S RNA respectively. The tall and short peaks on the electropherogram (b) correspond to 28S and 18S RNA respectively.

2.2.6.4 RNA quantification

A NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Leicestershire, UK) provided concentrations in ng/µl. The ratio of absorbance at wavelength of 260nm compared with 280nm (260/280) gave an indication of protein contamination. Values between 1.8 and 2.2 were deemed acceptable.

2.2.7 Reverse transcription

1µg of RNA was made up to 9.9µl with nuclease free water and denatured by incubating at 70ºC for 10 min in a thermal cycler. The Promega Reverse Transcription system (Promega, Southampton, UK) was used according to the manufacturer's instructions. Reagents and Random Primers were added to each sample in this order: 4µl of 25mM Magnesium Chloride, 2µl of Reverse Transcription 10X Buffer, 2µl of 10mM dNTP mixture, 0.5µl of Recombinant RNasin Ribonuclease inhibitor, 15u (0.6µl) of AMV Reverse Transcriptase (High Conc.) and 0.5µg (1µl) of Random Primers. The final volume was 20µl. Two negative controls were created: one with no RNA and the second with no AMV Reverse Transcriptase to ensure reagents were not contaminated and to detect genomic DNA. Samples were incubated at room temperature for 10 min for annealing and extension. Then using the thermal cycler, incubated at 42ºC for 15 min (for reverse transcribing), then at 95°C for 5 min (to inactivate enzymes) and finally cooled to 4ºC for 5 min. The resulting complementary DNA (cDNA) was stored at -20ºC.

2.2.8 Gene expression by qPCR

Relative expression of messenger RNA (mRNA) was quantified using a LightCycler 480 system (Roche, Burgess Hill, UK). Primers (detailed in Table 2.8) were a combination of inventoried assays (*Taq*man Gene Expression Assays, Applied Biosystems (ABI), Life Technologies, Paisley, UK) and custom made assays using the Universal Probe Library (UPL) (Roche, Burgess Hill, UK). The latter were designed to span intron boundaries. For analysing gene expression of *H19*, suitable primer sequences were obtained from Guo et al (Guo et al., 2008) and ordered from Invitrogen (Life Technologies, Paisley, UK) and the assay was carried out using

SYBR Green I technology. Primers were reconstituted to make 100µM stock solutions.

2µl from each cDNA sample were pooled together to make a stock solution and this was serially diluted to make a standard curve from 1:4 to 1:128. 10µl of the cDNA sample along with the two negative controls were diluted with 190µl of LightCycler H_2O (1 in 20 dilution). The standard curves were used to validate the primers and thereafter perform the assay. For the assay, the samples and the two negative controls were loaded in triplicate onto the 384 well plate for each gene.

2µl of cDNA was loaded into each well of a 384 well plate for each gene followed by 8µl of 'Master Mix'. For the *Taq*man assays, this comprised of 5µl of LightCycler 480 Probes Master, 2.5µl of LightCycler H_2O, and 0.5µl of the ready-made Primer Probe mix. For the UPL system, the 'Master Mix' comprised 5µl of LightCycler 480 Probes Master, 2.86µl of LightCycler H_2O, 0.02µl of forward primer, 0.02µl of reverse primer, and 0.1µl of the appropriate Probe (Roche Applied Science, West Sussex, UK). For the *H19* assay, the 'Master Mix' comprised 5µl of LightCycler 480 SYBR Green I Master, 0.05µl of forward primer, 0.05µl of reverse primer, and 2.9µl of LightCycler H_2O.

The reaction conditions were: pre-incubation (95ºC for 5 min), 50 amplification cycles (95ºC for 10 s for denaturing, 60ºC for 30 s for annealing, 72ºC for 1 s for elongation) and cooling (40ºC for 30 s). The reaction conditions for SYBR Green were: pre-incubation (95ºC for 5 min), 50 amplification cycles (95ºC for 10 s for denaturing, 62ºC for 20 s for annealing, 72ºC for 30 s for elongation), followed by a melt curve and then cooling (40ºC for 10 s).

The concentrations of the PCR products were calculated from the standard curve. The 'concentration' of mRNA for each sample was calculated as a mean of the triplicate assay relative to the mean of the reference gene.

	Gene name	ABI assay ID	
a	11 Beta hydroxysteroid dehydrogenase type 2 (*HSD11β2*)	Hs00388669_m1	
	Cyclin-dependent kinase inhibitor 1C (*CDKN1C*)	Hs00178938_m1	
	Delta like homolog 1 (*DLK1*)	Hs00171584_m1	
	Growth factor receptor-bound protein 10 (*GRB10*)	Hs00959286_m1	
	Nuclear receptor subfamily 3, group C, member 1 (*NR3C1*)	Hs00353740_m1	
	Insulin like growth factor 2 (*IGF2*)	Hs00171254_m1	
	Paternally expressed gene 10 (*PEG10*)	Hs00248288_s1	
	Pleckstrin homology-like domain, family A, member 2 (*PHLDA2*)	Hs00169368_m1	
	Peroxisome proliferator-activated receptor-gamma (*PPARγ*)	Hs01115513_m1	
	Zinc finger gene 2 (*ZIM2*)	Hs00377844_m1	
		Primer sequences	**UPL probe**
b	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC	60
	Insulin like growth factor 2 receptor (*IGF2R*)	F: AGCAGCAGGAAGATACCACAA R: CACCTCACCAATATATCAAGGTGA	58
	Succinate dehydrogenase complex subunit A (*SDHA*)	F: GGACCTGGTTGTCTTTGGTC R: CCAGCGTTTGGTTTAATTGG	80
	TATA box binding protein (*TBP*)	F: CCCATGACTCCCATGACC R: TTTACAACCAAGATTCACTGTGG	51
	Tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, zeta polypeptide (*YWHAZ*)	F: GATCCCCAATGCTTCACAAG R: TGCTTGTTGTGACTGATCGAC	30
c	Maternally expressed non-coding transcript (*H19*)	F: AACCCACAACATGAAAGAAATGG R: AGAGGGTTTTGTGTCCGGATT	-

Table 2.8 Primer sequences for gene expression

(a) ABI (b) UPL (c) Sequences for use with SYBR green. Abbreviations: F - forward; R – reverse

2.2.9 Reference genes

Endogenous reference genes are used to normalise gene expression levels allowing comparison across samples and the use of internal controls ('housekeeping genes') is the most common method. A pre-requisite for a suitable reference gene should be to demonstrate adequate expression levels and show minimal variability between the normal and experimental conditions. The utility of the reference gene must be experimentally validated for a particular tissue type and the specific experimental design as physiological and pathological processes can influence expression (Bustin et al., 2009). This is particularly applicable to human studies where homogeneity of participants and conditions cannot be guaranteed unlike in animal models, and where the tissues are subject to variation in collection and prone to degradation. The human placenta typifies this scenario: following delivery, the immediate attention is to the mother and newborn and sampling from the placenta may be delayed. This can lead to degraded RNA and artificial changes in gene expression (Lanoix et al., 2012; Vermeulen et al., 2011). Furthermore, gender may influence the expression of housekeeping genes in the placenta (Cleal et al., 2010).

The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) Guidelines recommend objectively evaluating reference genes (Bustin et al., 2009) and the search for the optimal reference gene in the placenta has been the subject of a few studies using geNorm (http://medgen.ugent.be/wjvdesomp/genorm/, accessed 11 Apr 2011) software . Out of 7 candidates, TATA box binding protein (*TBP*) and succinate dehydrogenase complex subunit A (*SDHA*) were found to be the most stable followed by Tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, zeta polypeptide (*YWHAZ*) (Meller et al., 2005). In a similar study, ubiquitin C (*UBC*) and topoisomerase DNA (*TOP1*) appeared to be the most stable, followed by *YWHAZ* (Cleal et al., 2009). Comparing placental samples from late preterm pregnancies with fetal growth restriction with gestation matched controls, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and 18S ribosomal RNA (*18S rRNA*) showed the most

stability again followed by *YWHAZ* (Murthi et al., 2008). Hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*), peptidylprolyl isomerase A (*PPIA*) and *TOP1* were the most stable when comparing normotensive and pre-eclamptic pregnancies whereas *PPIA*, *YWHAZ* and *GAPDH* were optimal for comparing normal pregnancies with gestational diabetes (Lanoix et al., 2012) indicating that pathological processes affect reference genes. This study then went on to show that the choice of reference gene affected the gene expression result significantly (Lanoix et al., 2012).

Based on the above results and availability of primer sets in the laboratory, I therefore chose to assess the stability of *TBP, SDHA, YWHAZ* and *GAPDH* in the placenta samples using geNorm^{PLUS} as part of the qbase^{PLUS} software (Eclipse v 3.7), the updated software available from Biogazelle (Zwijnaarde, Belgium) at the time. The premise of the geNorm^{PLUS} program is that the ratios between stably expressed genes should remain regular. The program calculates *M,* which is the average pair-wise variation of a single reference gene from the raw expression data. The highest *M* value denotes the gene with the most instability (*TBP* in this study) and this is removed at each step. Thereafter, a new *M* value is calculated for the remaining genes until only 2 genes remain viz. *GAPDH* and *YWHAZ* in this study, which cannot be resolved from each other (Vandesompele et al., 2002) (Figure 2.2). The MIQE Guidelines define reference gene stability with *M* values below a threshold of 1.0 for heterogeneous samples and below 0.5 for homogeneous samples (Bustin et al., 2009). Both *GAPDH* and *YWHAZ* had *M* values of 0.98, just below the threshold for heterogeneous samples.

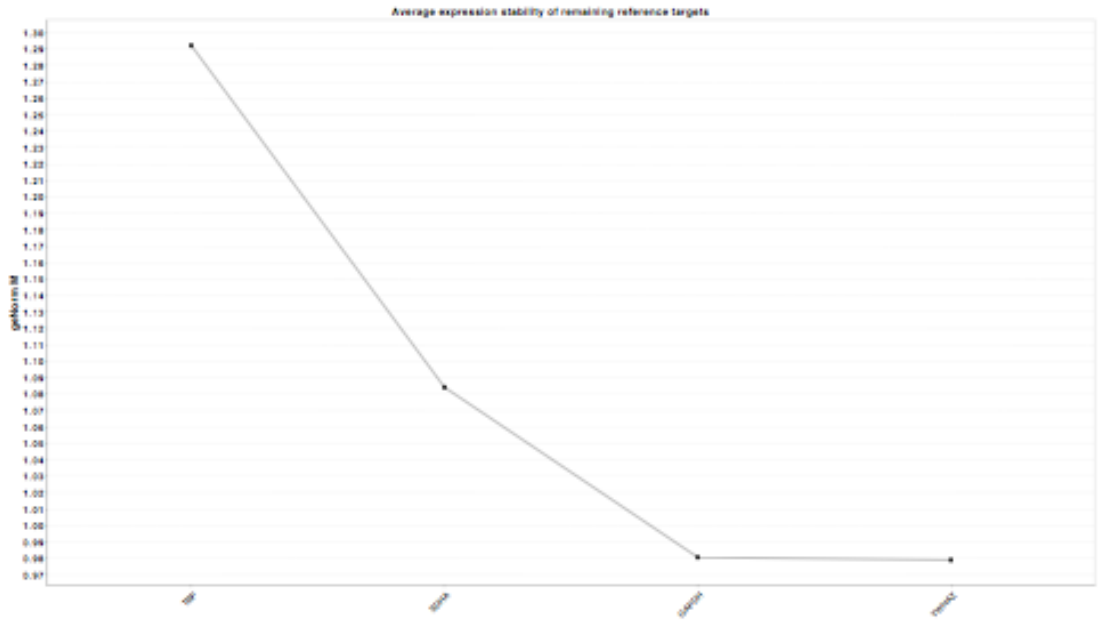


Figure 2.2 geNorm^{PLUS} output depicting stability of genes

The candidate genes were further assessed by NormFinder, a Microsoft Excel add-in freely available on the internet (http://www.mdl.dk/publicationsnormfinder.htm, accessed 24 Aug 2011) (Andersen et al., 2004). The algorithm ranks the set of candidate reference genes according the their expression stability in a given sample and given experimental design. The algorithm estimates the overall expression variation but also the variation between sample subgroups of the sample set. A low stability value indicated high intra- and inter-group expression stability. *SDHA* had the lowest stability value despite a high *M* value according to geNorm^{PLUS} (Table 2.9). Differences in results between geNorm and NormFinder where also found by Lanoix *et al* and *SDHA* was also inconsistent (Lanoix et al., 2012). Considering the two outputs, *YWHAZ* was chosen as the reference gene.

Gene name	**Stability value**	**Best gene**	SDHA
TBP	0.131		
GAPDH	0.103		
SDHA	0.079		
YWHAZ	0.087		

Table 2.9 NormFinder output indicating stability values of reference genes

2.2.10 Assessment of 5mC and 5hmC by qPCR

The process requires RNA free DNA to be sheared to the appropriately sized fragments. Capturing 5mC is based on the Methylated CpG Island Recovery Assay (MIRA) which utililises the high affinity of the methyl binding domain MBD2b/MBD3L1 (methyl binding domain) protein complex for 5mC. DNA with 5hmC is immunoprecipitated by a rabbit polyclonal antibody against 5hmC. The remainder of the techniques for the enrichment of 5mC and 5hmC are similar. Protein-DNA/antibody-DNA complexes are then captured with nickel-coated magnetic beads and subsequent wash steps remove fragments with little or no methylation. Proteinase K is then used to cleave off the DNA off the beads and the DNA is eluted. Percentage enrichment of 5mC and 5hmC at regions of interest were studied by qPCR. A pictorial overview of the procedure is in Figure 2.3.

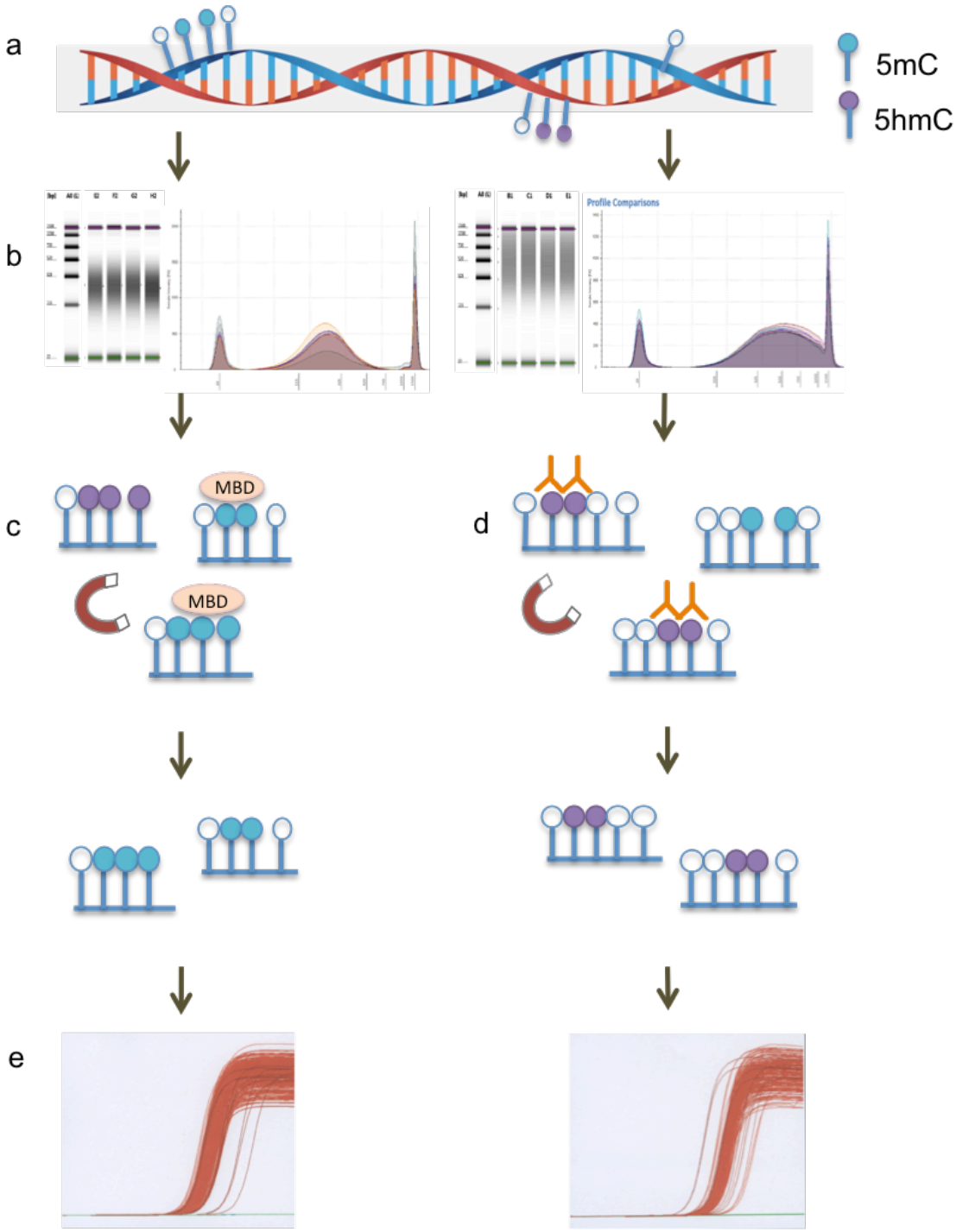


Figure 2.3 The 5mC and 5hmC assay

a) Genomic DNA b) shearing to fragments by sonication c) capturing DNA fragments with 5mC using MBD protein d) immunoprecipitation of DNA fragments with 5hmC e) qPCR to study regions of interest.

2.2.10.1 DNA extraction from placenta

DNA extraction was carried out using DNeasy Blood & Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions (November 2006 edition) and with modifications. All centrifugation was done at room temperature.

25mg of placental tissue was placed in 360µl Buffer ATL with 40µl Proteinase K (23mg/ml) and incubated at 56°C overnight in a hybridisation oven. (The suggested volumes for both Buffer ATL and Proteinase K were doubled to increase DNA yield). 15µl of RNase A (10mg/ml) (Sigma Aldrich, UK) was added, vortexed to mix and incubated for 2 min at room temperature (the suggested amount of 40µl of 10mg/ml RNase A was reduced in steps and 15µl was chosen to yield RNA free DNA whilst minimising the possibility of reducing total DNA yield). After a further vortex for 15 s, 400µl of Buffer AL plus 400µl 100% ethanol (pre-mixed) was added and mixed by vortexing (the suggested volumes for both Buffer AL and 100% ethanol were doubled to increase DNA yield). This mixture was transferred into a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 min. The flow through was discarded. The column was placed in a new collection tube, 500µl Buffer AW1 was added, centrifuged at 8000 rpm for 1 min, flow through and collection tube discarded. The column was placed in a new collection tube, 500µl Buffer AW2 added, centrifuged at 14,000 rpm for 3 min to dry the membrane and the collection tube with the flow through discarded. 100µl of Buffer AE was transferred directly onto the membrane, incubated at room temperature for 1 min and centrifuged at 8000 rpm for 1 min to elute the DNA. The final step was repeated to increase overall DNA yield.

2.2.10.2 DNA quality assessment

DNA quality was assessed according to the method described in 2.2.3.1. Additionally, the aim was to show no products suggestive of RNA on the gels. If RNA was found, the sample was discarded and DNA extraction repeated from a new piece of tissue.

2.2.10.3 DNA quantification

DNA was quantified according the method described in 2.2.6.4 using the NanoDrop Spectrophotometer. Additionally, DNA was quantified using the Qubit 2.0 Fluorometer according the method described in 2.2.3.2. This technique utilises fluorescence-based dyes that bind specifically to DNA and thus accurately measures the concentration of DNA whereas the NanoDrop does not discriminate between DNA and RNA in the same sample. The measured concentrations of the DNA samples by the Qubit approximated those by the NanoDrop further indicating that the DNA was free of RNA. The value obtained by the Qubit was used for calculations.

2.2.10.4 Sonication

Initial sonication was using a random selection of 12 DNA samples using the Bioruptor ultrasonic disruptor (Diagenode, Liège, Belgium). Briefly, 5µg of each sample was halved. Each 2.5µg of DNA was made up to 200µl with Buffer AE and sonicated for 30 s on and 30 s off at 4°C. The sonication was for cycles of 8 to 15 to achieve, for one half the samples, DNA fragments with a mean size of 200 to 300bp and for the other half, mean size of 500 to 600bp. 5µl of each sample was electrophoresed for ~ 45 min at 140 volts on a 1.2% agarose gel to check the fragments at intervals until the desired size was achieved.

The Covaris E220 ultrasonicator with Adaptive Focused Acoustics (AFA) (Covaris, MA, USA) was used for the remainder of the samples as it allowed for higher throughput and fidelity. 2.5µg of DNA in 100µl Buffer AE was transferred to Crimp-Cap microTUBEs with AFA fibre via the pre-split septum taking care to avoid bubbles and the tubes loaded onto the Covaris rack. Initially, surplus DNA was used to test variations on the manufacturer's recommended acoustic parameters to achieve the desired mean fragment sizes. Increasing the duration, the duty factor and the peak incident power resulted in smaller fragments. Ten different variations were tested in total with the following parameter ranges: time (60 – 180s), Peak Incident Power (105 – 175 Watts) and Duty Factor either 5 or 10%. The Cycles per Burst was kept constant at 200. To achieve a peak between 200 and 300bp, the sonication was for 180 s with a Peak Incident Power of 175 Watts and a Duty Factor of 10%. To

achieve a peak between 500 and 600bp, the sonication was for 65 s with a Peak Incident Power of 105 Watts and a Duty Factor of 5%.

Following the sonication, the samples were removed from the microTUBEs and sonication profiles were measured using the Agilent 2200 TapeStation Instrument (Agilent Technologies, Cheshire, UK) with the D1K ScreenTape assay according to the manufacturer's instructions. The principle of the technique is similar to that described in 2.2.6.3. Electropherograms depict the distribution of the fragment sizes and indicate the peak value. Following batch sonication of the samples, 15 were randomly selected for quality assurance using the TapeStation. The amalgamated electropherograms showed that the shearing by the Covaris is reproducible (Figure 2.3 (b)).

2.2.10.5 Capture of 5mC by methyl binding domain (MBD) protein

The Active Motif MethylCollector Ultra Kit (catalogue #55005) (was used and the manufacturer's instructions were followed. Sample DNA can amount from 1ng to 1µg. A range of sample DNA amounts was tested and 500ng was chosen to yield quantifiable amplification by qPCR whilst conserving DNA. DNA that had been sonicated to a peak fragment size of 200 to 300bp was required. 10% of this DNA i.e. 5ng was kept aside as the 'input' and made up to 10µl with Buffer AE. To make a binding reaction, the following were added to a PCR tube in order: 10µl Magnetic beads, 70µl Complete Binding Buffer (Low Salt Binding Buffer plus Protease Inhibitor Cocktail), 500ng DNA and 10µl His-MBD2/MBD3L1 protein complex. After mixing, the tube was incubated on an orbital shaker (Stuart Rotator SB2, Biby Sterilin LT8, Stone, UK) at 4°C for 1 h. The PCR tube was centrifuged briefly, placed on the magnetic strand to pellet the beads and the supernatant discarded. The beads were washed 4 times with 200µl Binding Buffer, each time the beads were re-suspended by pipetting several times and the supernatant removed by using the magnetic strand to pellet the beads. After the final wash and the supernatant removed, the beads were re-suspended in 100µl Complete Elution Buffer (2µl Proteinase K added to 98µl Elution Buffer) by pipetting 2-3 times and incubated at 50°C for 30 min in a hybridisation oven. The beads were pelleted using the magnetic strand, the supernatant with methylated DNA fragments transferred to new a tube

and 2µl of Proteinase K Stop Solution (previously warmed to 37°C on a hot block for 10 min) added and pipetted 2-3 times to mix.

2.2.10.6 Immunoprecipitation of 5hmC

2.5µg of DNA with peak fragment size 500 to 600bp was made up to 450µl with Buffer AE, denatured for 10 min in boiling water on a heat block and then immediately cooled on ice water for 5 min. 50µl of 10x IP buffer was added (then 50µl was removed (i.e. 10%) and set aside from this procedure as the 'input') and DNA immunoprecipitated with 2µl of a rabbit polyclonal antibody against 5hmC (Active Motif, catalogue #39791) at 4°C for 2 h. 40µl of magnetic M-280 protein G Dynabeads (Invitrogen #100-03D) was pre-washed with 800µl PBS-BSA 0.1% with shaking (Stuart Gyro-rocker SSL3, Bibby Scientific, Staffordshire, UK) at room temperature. The beads were collected using a magnetic rack (Dynal MPC-S Magnetic Particle Concentrator, Dynal Biotech, Norway), re-suspended in 40µl of 1x IP buffer and added to the DNA-5hmC mix. The resulting mixture was incubated at 4°C for 1 h on the orbital shaker prior to washing all unbound fragments three times with 1ml 1x IP buffer. The first wash was with cold IP buffer; the second and third washes were with IP buffer at room temperature. Each wash was for 10 min on an orbital shaker. Washed beads were re-suspended in 250µl Proteinase K digestion buffer, 10µl Proteinase K (20mg/ml) added and incubated at 50°C with mixing (Thermomixer, Eppendorf, Hamburg, Germany) set at 400 rpm overnight. Samples were briefly centrifuged at 2000 rpm and beads removed with the magnetic rack.

2.2.10.7 Purifying DNA fragments

The samples with DNA fragments pulled down by MBD protein and immunoprecipitated DNA fragments along with the corresponding 'input' samples were then purified. The QIAquick PCR Purification Kit (Qiagen, Crawley, UK) was used and the manufacturer's instructions were followed (March 2008 edition). All centrifugation steps were carried out at 13,000 rpm at room temperature.

5 volumes of Buffer PB (with 1:250 volume of pH indicator I added to increase DNA yield) were added to 1 volume of DNA sample. If the resulting mixture did not turn yellow, then 10µl of 3M sodium acetate pH 5.0 was added and mixed. To bind the

DNA, the sample was applied to the QIAquick column in a collection tube, centrifuged for 60 s and the flow-through discarded. The column was washed with 750µl Buffer PE and centrifuged for 60 s and the flow-through was discarded. The centrifugation was repeated to remove residual ethanol and the column was placed in a clean microcentrifuge tube. To elute DNA, 50µl Buffer EB was applied directly to the membrane, allowed to stand for 1 min and then centrifuged for 1 min. The eluate was further diluted with Buffer EB in a ratio of 1:3.

2.2.10.8 Analysis of percentage enrichment by qPCR

Percentage enrichment of 5mC and 5hmC was quantified using the LightCycler 480 system (Roche, Burgess Hill, UK). Primers (detailed in Table 2.10) were obtained from Invitrogen (Life Technologies, Paisley, UK), reconstituted to make a 100mM stock solution and carried out using SYBR Green I technology.

Randomly chosen sonicated DNA samples that were surplus were pooled to 200µl and serially diluted in Buffer AE to create a standard curve from 1:10 to 1:1000. The standard curves were used to validate the primers and thereafter perform the assays. For each assay, the samples and a negative control were loaded in triplicate and the corresponding 'input' samples and negative control were loaded in duplicate onto the 384-well plate. This compromise was to ensure that one plate was used for each assay.

2µl of sample was loaded into each well followed by 8µl of 'Master Mix'. This comprised of 5µl of LightCycler 480 SYBR Green I Master, 0.025 - 0.05µl of the forward primer, 0.025 - 0.05µl of the reverse primer, and 2.9 - 2.95µl of LightCycler H_2O. The amount of primers was halved in some instances to diminish the formation of primer dimers. The reaction conditions were: pre-incubation (95ºC for 5 min), 50 amplification cycles (95ºC for 10 s for denaturing, 62ºC for 20 s for annealing, 72ºC for 30 s for elongation), followed by a melt curve and then cooling (40ºC for 10 s).

The concentrations of the PCR products were calculated from the standard curve. The percentage enrichment of 5hmC or 5mC each sample was calculated as the mean of the sample assay relative to the mean of the assay for the corresponding 'input'

sample (multiplied by 10) and further multiplied by 100. Percentage enrichment of 5hmC and 5mC at the GAPDH promoter was assayed as a negative control and there were very low levels of enrichment of 5hmC and no amplification in many instances for 5mC. Therefore GAPDH was not used as a reference gene.

Primer name	Primer sequences	Chromosome location
GAPDH[1]	F: CGGCTACTAGCGGTTTTACG R: AAGAAGATGCGGCTGACTGT	chr12:6513796+6513984
H19 promoter[1]	F: CCTGGAATTCTCCAAAGACG R: AGTGGTCTGGGAGGGAGAAG	chr11:1975813+1975927
H19 genic 1[1]	F: CTCAGCTCTGGGATGATGTG R: AGCCCAACATCAAAGACACC	chr11:1973443+1973573
H19 genic 3[1]	F: CTGGTGCTCACCTTCCAGAG R: ATGGTGCTACCCAGCTCAAG	chr11:1973671+1973802
H19 genic 4[1]	F: GCCAGCTACACCTCCGTTG R: AGCTAGGGCTGGAAAGAAGG	chr11:1975242+1975378
H19 DMR[1]	F: GATCTCGGCCCTAGTGTGAA R: GTGATGTGTGAGCCTGCACT	chr11:1977756-1977943
H19 CTCF3[2]	F: CACACCACGTCTTCGTATCG R: ATTTGGGGGCTGTCCTTAGT	chr11:1979701-1979890
H19 CTCF6[2]	F: AGTTGTGGAATCGGAAGTGG R: GATAATGCCCGACCTGAAGA	chr11:1977617-1977807
IGF2 DMR0[2]	F: TTTCATATTCCGTGCCATGA R: CCTGCCTAGAGCTCCCTCTT	chr11:2125774-2125987
IGF2 DMR2[2]	F: CGTTGAGGAGTGCTGTTTCC R: CACAGCAAGCAAGGAAGTCA	chr11:2111194-2111431
KvDMR[3]	F: TGCGGATTCCAGACTCCAAT R: GCTCCCATCTGCACCTTATG	chr11:2676925+2677065

Table 2.10 Primer sequences for 5mC and 5hmC qPCR

Chromosome location refers to UCSC Genome Browser hg18. Primer sequences obtained from 1) Nestor et al (Nestor et al., 2012) 2) AJ Drake 3) designed by myself.

2.3 Statistics

All statistical analysis was using SPSS for Mac v19 (IBM, New York, USA). Independent and paired samples t tests were used to compare continuous variables between groups and multivariate linear regression was used to adjust for confounders. Paired samples t tests were used where appropriate. The chi square test was used for categorical variables. Pearson's correlation coefficient was used to measure relationships between normally distributed variables and multivariate linear regression was used to adjust for confounders. Further details are given in each experimental chapter.

All graphs were created using Prism v5.0b for Mac OS X (GraphPad Software Inc, CA, USA).

Weight, head circumference, length, weight gain, subscapular skinfold and triceps skinfold measurements were converted to standard deviation scores using LMSgrowth version 2.71. This is a Microsoft Excel Add-in to access growth references based on the LMS method provided by the Child Growth Foundation (http://www.healthforallchildren.com/?product=lmsgrowth, accessed 7 February 2011). They define the UK-WHO growth charts.

The following sources were used:

"UK_WHO_term.xls" includes values for term birth at 37-42 weeks computed from the "British 1990 reference data, reanalysed 2009". This is combined with the "WHO Child Growth Standards" from 2 weeks to 4 years. From 4 years, the data is from the "British 1990 reference".

"UK_WHO_preterm.xls" is identical the above except that it includes values for births from gestations 23 to 42 weeks, which is also from "British 1990 reference data, reanalysed 2009".

"WHO2006.xls" includes data for subscapular and triceps skinfold for 0.25-5 years.

2.4 Software

SPSS for Mac v19 (IBM, New York, USA)

Prism v5.0b for Mac OS X (GraphPad Software Inc, CA, USA)

PyroMark Q24 Software 2.0 (Qiagen, Crawley, UK)

NormFinder Microsoft Excel 2003 Add-in v0.953

geNorm^{PLUS} (qbase^{PLUS} Eclipse v3.7, Biogazelle, Zwijnaarde, Belgium)

2100 Expert Software (Agilent Technologies, Cheshire, UK)

LightCycler 480 release 1.5 O SP3 (Roche, Burgess Hill, UK)

ND-1000 v3.3 (NanoDrop Technologies, Thermo Fisher Scientific, Leicestershire, UK)

SoftMax Pro 5.0 Microplate Data Acquisition and Analysis (Molecular Devices Ltd, Berkshire, UK)

2.5 Funding

The studies were funded by the Chief Scientist's Office for Scotland.

Chapter 3: Programming in the preterm

3.1 Introduction

Preterm infants are at increased risk of the metabolic syndrome in later life (Parkinson et al., 2013) (Tinnion et al., 2014). The mechanisms accounting for such 'programming' effects are unclear, but may include extra-uterine growth restriction followed by catch-up growth (Fewtrell et al., 2000) (Rotteveel et al., 2008) and abnormal HPA axis activity (Grunau et al., 2007) (Reynolds et al., 2001).

Changes to the epigenome may be one molecular mechanism mediating such programmed effects. Early life stress in the form of exposure to an unbalanced diet or famine during gestation has been associated with cardio-metabolic outcomes in the offspring as adults, along with altered DNA methylation at the *H19 ICR* (Drake et al., 2012) and *IGF2 DMR0* and *DMR2* (Tobi et al., 2012) (Heijmans et al., 2008). Altered DNA methylation at the *IGF2 DMR0* has also been measured in adults born preterm (Wehkalampi et al., 2013). However it is not known whether differences such as these would have been present and identifiable at birth or whether they develop later in life. By virtue of being an imprinted region, *IGF2/H19* is developmentally regulated by DNA methylation and can be susceptible to environmental insults. Given that changes in DNA methylation at this locus are thought to be stable over decades (Heijmans et al., 2007) and that changes to the methylome have been identified with respect to gestation (Schroeder et al., 2011), *IGF2/H19* is a candidate for study of DNA methylation in preterm infants.

3.2 Aims and Hypothesis

I aimed to (1) recruit a cohort of preterm infants and term controls (2) measure growth (including body composition) and HPA axis activity and (3) test the hypothesis that preterm infants have altered DNA methylation (5mC) at *IGF2/H19* over the first year of life compared to term infants.

3.3 Methods

3.3.1 Subjects

Following ethical approval and written parental consent, preterm and term infants were recruited within the first week of life. Demographic details and history were obtained following consent, during clinic visits and from the mother's hospital records.

Infants were seen within the first week of life ("birth"), 3 months/3 months corrected for preterm infants ("3 months") and 1 year/1 year corrected age ("1 year"). Additionally, the preterm infants were seen at postmenstrual age of 37 to 42 weeks (denoted as "term corrected age"). All time points mentioned henceforth refer to the corrected age denoted within brackets for simplicity.

3.3.2 Clinical assessments

Weight, length and occipito-frontal circumference (OFC) was measured at all time points.

Percentage body fat mass was measured by air displacement plethysmography (densitometry) using the PEAPOD Body Composition System at birth/term corrected age and 3 months. Skin fold thickness was measured at 1 year.

Saliva for cortisol was collected at 3 months at the end of the clinical examination. All clinic visits were scheduled in the afternoon for consistency.

3.3.3 Laboratory methods

Salivary cortisol was measured by the High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit.

Saliva for buccal DNA was collected using Oragene DNA (OG-250) kits. DNA was extracted following the manufacturer's guidance. Percentage methylation was analysed using Pyrosequencing after bisulphite conversion.

3.3.4 Statistics

Weight, length, OFC, skin fold thickness measurements and weight gain were adjusted for age and gender by converting to standard deviation (SD) scores (z-scores) using LMSgrowth, a Microsoft Excel Add-in to access growth references that define the UK-WHO growth charts.

Independent samples t testing was used to test the differences in growth attainment and cortisol between term and preterm infants. Multivariate linear regression was used to assess differences in body composition. Multivariate linear regression analysis was also used to test the hypothesis that preterm birth is associated with altered DNA methylation. Covariates that could confound the association or be in the causal pathway were added into the model in a hierarchical manner. Outcome variables were percentage DNA methylation at birth, term corrected age and 1 year. Also included were variables that were grossly unequal between the groups: breast milk at 3 months (entered into the model for analyses beyond the neonatal period) and mode of delivery (entered into the model for analyses in the neonatal period). The unstandardised regression (β) coefficients from these models indicate the number of units that percentage DNA methylation will change as a result of one unit change in the other predictors. Paired samples t testing was used where appropriate. Pearson's correlation coefficient to test for relationships between normally distributed variables and Spearman's rho for ordinal values.

Since in general, neighbouring CpG sites tend to have similar levels of methylation (Nautiyal et al., 2010), percentage DNA (cytosine) methylation (%5mC) is expressed as the average across all CpG sites in the locus tested rather than at individual CpG sites and analysed as such.

Salivary cortisol values were log transformed using natural logs to achieve a normal distribution. All other values were normally distributed or converted to standard deviation scores.

Based on a previous study in this department, 25 infants were needed in each group to detect a 5% difference in DNA methylation (Drake et al., 2012). We obtained approval to recruit 50 in each group to account for drop out.

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$ (2 tailed).

3.3.5 Covariates

Social deprivation was coded as DEPCAT (deprivation category) scores. These are based on the mother's postcode at booking and obtained from "Carstairs scores for Scottish postcode sectors from the 2001 Census" (McLoone, 2004). The scores are from 1 to 7 where 7 indicates the worst social deprivation. *Maternal smoking* was categorised as current smoker, never smoked, former (stopped pre-pregnancy) or former (stopped during pregnancy). *Labour* was whether the mother experienced labour prior to delivery or not. *Breast milk at 3 months* indicates whether or not the infant was receiving any breast milk at 3 months corrected age.

3.4 Results

3.4.1 The cohort

Forty infants at full term were recruited. Five of the term infants were admitted to the neonatal unit for problems with transitioning: respiratory distress syndrome, excessive early weight loss, and haemolysis from Rh isoimmunisation. The average length of stay was 4.5 days, range of 2 – 9 days. None were routinely followed up in the neonatal clinic.

Fifty preterm infants were recruited. Four of these infants died in the neonatal period and buccal DNA was not obtained for analysis. In three infants, saliva for buccal cells was not collected in view of the severity of their clinical condition and in the fourth infant, the DNA sample was of very poor quality. Therefore 46 preterm infants were included for analysis. There were two further deaths in the neonatal period, however buccal cells were collected after birth and the DNA was suitable for analysis. The synthetic steroid available to the obstetricians during the period of recruitment (to help fetal maturation when preterm delivery was threatened) was

dexamethasone due to a national shortage in the supply of betamethasone. None of the preterm infants received postnatal steroids for hypotension or for dependence on mechanical ventilation.

	Term	Preterm
Gestation at birth, weeks	40.2 (38+3-42.2)	28.8 (25+2–31+5)
Birth weight, g	3649 (2640–4740)	1174 (550–1820)
Birth weight SDS	0.44 (-1.52–2.92)	-0.42 (-3.15–1.05)
Male, *n* (%)	15 (38)	31 (67)
Bronchopulmonary dysplasia, *n* (%)	0	13 (30)
Laser for retinopathy of prematurity, *n* (%)	0	4 (9.1)
Necrotising enterocolitis, *n* (%)	0	6 (13.3)
Intraventricular haemorrhage, *n* (%)	0	1 (2.2)
Periventricular leucomalacia, *n* (%)	0	2 (4.5)
Late onset sepsis, *n* (%)	0	17 (37)
TPN duration, days	0	14.9 (0–107)
Any breast milk at 3 months, *n* (%)	28 (75.7)	9 (22.5)

Table 3.1 Characteristics of the study participants

Values are means (range). $N = 40$ full term and 46 preterm infants. Birth weight SDS was different between the groups, $p < 0.001$ (independent samples t test) and the frequency of male gender was different between the groups, $p = 0.006$ (Pearson Chi square test). Bronchopulmonary dysplasia (BPD) is defined as need for respiratory support and/or supplemental oxygen at 36 weeks' postmenstrual age to maintain oxygen saturations of 90% or more. A diagnosis of necrotising enterocolitis was applied when cases achieved Bell stage 2 or greater. Intraventricular haemorrhage is defined as only grade III or IV events. The definition of late onset sepsis is taken from the Vermont Oxford Network Manual of Operations, release 16.3. Missing data: 2 preterm infants died before an age where retinopathy of prematurity develops or a diagnosis of BPD is given. Three term infants and 6 preterm infants were not seen at 3 months. Abbreviation: SDS – standard deviation score.

	Term	Preterm
Age, years	35.2 (25.5-44.2)	31.3 (20 –41.4)
BMI at booking, kg/m^2	24.3 (19.1-33.5)	27.2 (17.9-46)
DEPCAT score, mode	3	4
Caucasian ethnicity, *n* (%)	40 (100)	38 (92.7)
Smoking, *n* (%)		
Current	0	8 (19.5)
Former – stopped during pregnancy	2 (5)	4 (9.8)
Former - stopped pre-pregnancy	12 (30)	11 (26.8)
Never	26 (65)	18 (43.9)
Primiparity, *n* (%)	21 (52.5)	25 (61)
Folic acid during first trimester, *n* (%)	40 (100)	35 (94.6)
Assisted reproduction, *n* (%)	0	6 (14.6)
Multiple pregnancy, *n* (%)	0	8 (19.5)
Hypertension or pre-eclampsia, *n* (%)	0	10 (24.4)
Diabetes during pregnancy, *n* (%)	0	3 (7.3)
Antenatal steroids, *n* (%)		
None	40 (100)	3 (7.3)
Incomplete course	-	11 (26.8)
Complete course	-	27 (65.9)
Antenatal Magnesium sulphate, *n* (%)	0	9 (22)
Caesarean section, *n* (%)	28 (70)	23 (56.1)
Labour, *n* (%)	18 (37.5)	24 (58.5)
Age of partner	35.3 (26.7-46.2)	32.9 (21.9-46.7)

Table 3.2 Maternal characteristics of the study participants

Values are means (range). $N = 40$ mothers for full term infants and 41 for preterm infants. Abbreviation: DEPCAT – deprivation category. Maternal age and BMI were different between the two groups, $p = 0.002$ and $p = 0.017$ respectively (independent samples t test). Age of partner was not significantly different, $p = 0.07$.

3.4.2 Anthropometry in infancy

3.4.2.1 Longitudinal growth

Term infants were measured on average 2.1 days (range 0 to 8) after birth, at 3 months (13.3 weeks; range 10.4 to 16.9) and 1 year (52.6 weeks; range 48.1 to 57.4). Preterm infants were measured at term corrected age at an average of 39.3 (range 35+0 to 44+1) corrected weeks, at 3 months corrected (13.8 weeks; range 10.3 to 18.3) and 1 year corrected (54.4 weeks; range 52.1 to 68.7).

The mean birth weight SD score for the preterm cohort was -0.4 ± 0.9 however by term corrected age, the mean SD score for weight was -1.4 ± 0.2, indicating downward deviation from the birth SD score. In contrast, the mean SD score for head circumference in preterm infants at term corrected age was -0.2 ± 0.2. The significant difference in head circumference SD score at term corrected age is in the context of greater than average measurements in the term infants. (Figure 3.1) Growth restriction during the third trimester occurs as a result of factors such as placental insufficiency or maternal illness and there is a relative sparing of head growth (Kramer et al., 1989). Data suggests that this pattern of growth tends to persist in the neonatal unit, with sparing of head growth (Cockerill et al., 2006) resulting in asymmetric growth restriction at term corrected age (Uthaya et al., 2005).

Preterm infants were lighter and shorter than term infants at all comparative time points, $p < 0.05$. The exception to this is at 1 year corrected, where the preterm infants had a length measurement equivalent to that of term infants, $p = 0.26$. By 1 year, mean SD score for weight was -0.01 ± 0.2 in the preterm group. The differences later in infancy are mainly as the term infants are larger than the reference mean. In preterm infants, weight and length SD scores at term corrected age were significantly less than zero ($p <0.001$), as were weight and length SD scores at 3 months ($p <0.001$ and $p = 0.008$ respectively), but not at 1 year ($p = 0.89$ and 0.15 respectively). OFC SD scores at term corrected age and 3 months were not significantly different from zero ($p = 0.35$ and 0.15 respectively) but significantly larger than zero at 1 year ($p <0.001$) (Figure 3.1).

Although it therefore appears as though preterm infants exhibited ‘catch-up’ weight gain, there were no significant differences between preterm and term infants in mean weight gain SD scores between birth/term corrected age and 3 months (mean difference -0.02, 95% confidence interval (CI) [-0.6, 0.5], t =-0.1, $p = 0.93$). Nor was there a significant difference in mean weight gain SD scores between 3 months and 1 year (mean difference -0.2, 95%CI [-0.7, 0.2], t = -1.1, $p = 0.29$). In other words, the preterm infants did not have greater velocity of weight gain than the term infants (Figure 3.2).

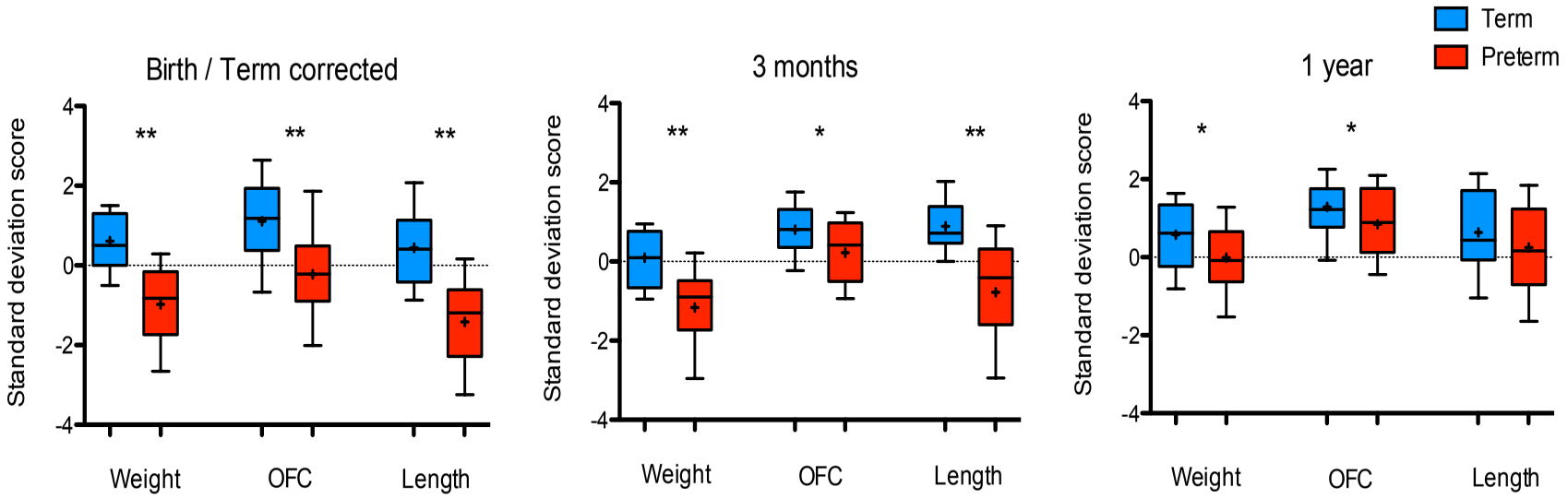


Figure 3.1 Growth attainment of term and preterm infants over the first year

Box and whisker plot: 10th to the 90th percentile with the line at median (+ indicates the mean). Term infants: $n = 40$ at birth, 35 at 3 months and 35 at 1 year. Preterm infants: $n = 43$ at term corrected, 35 at 3 months and 42 at 1 year. *$p < 0.05$ **$p < 0.001$

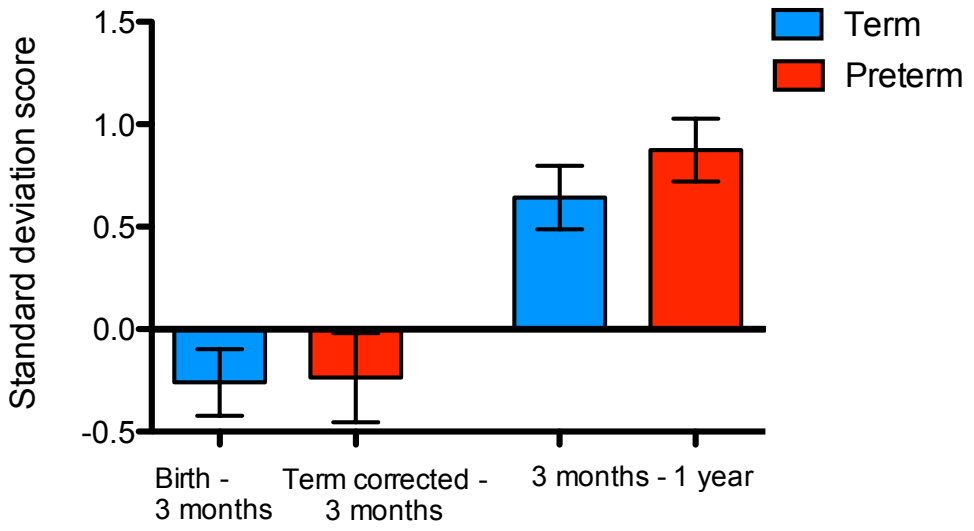


Figure 3.2 Change in weight SD score between term and preterm infants over the first year

Mean ± SEM. Term (birth to 3 months), n = 35, (3 months to 1 year), n = 33. Preterm (term age to 3 months), n = 36, (3 months to 1 year) n = 34.

3.4.2.2 Body composition

Term infants had body composition measured on average 2 days (range 0 to 7) after birth and again at 3 months (13.2 weeks; range 10.4 to 16.9). Preterm infants had body composition measured at average 39.8 weeks post-menstrual age (range 36+2 to 42+4) and again at 3 months (13.8 weeks; range 10.6 to 18.3). Not all infants recruited were assessed. For the term infants, 8 parents declined for assessment after birth. At 3 months, 2 parents declined, the PEAPOD was out of order for 2 infants, 1 infant was in a Pavlik harness for developmental dysplasia of the hips and 5 infants were not seen. For the preterm infants at term equivalent age, 8 infants were receiving respiratory support for BPD and 4 were dependent on parenteral nutrition on the neonatal unit. Many who do well are discharged either home or to their local neonatal unit well before term age, thus measurements at term corrected age were not obtained for 11 such infants. This was recognised as the study was conducted and parents were invited to return for assessment. Assessments obtained > 36 weeks corrected age was used for analysis. At 3 months, 2 parents declined, 1 infant was dependent on parenteral nutrition and remained an inpatient, 8 were not seen and in

1, the measurement was aborted accidentally and not repeated as the infant was distressed. The numbers studied at each time point are shown in the graphs.

Preterm infants had a higher percentage body fat (17.1 ± 1.0%) at term corrected age when compared with term infants at birth (11.6 ± 0.7%). The difference, 5.5%, 95%CI [8.0, 3.0], was significant $t = 4.4$, $p < 0.001$ and remained so with adjustment for gender, $\beta = 5.7$, 95% CI [3.1, 8.3], $p < 0.001$.

This difference in percentage body fat did not persist at 3 months, (preterm infants 23.7 ± 0.7%; term infants 24.6 ± 0.7%). This remained so after adjustment for gender and breast milk intake ($\beta = -0.3$, 95% CI [-2.8, 2.2], $p = 0.82$) (Figure 3.3). This was despite male gender appearing to lower percentage fat mass in this model. Table 3.3 details the regression coefficients for the covariates. Although some previous studies in full term breast fed or formula fed infants have not shown a difference with gender (Roggero et al., 2010) (Eriksson et al., 2010) (Carberry et al., 2010), others have shown that although gender did not affect fat mass in preterm infants, but it did so in full term infants (Simon et al., 2013). In our study, adjustment for gender was undertaken in view of the large disparity between the two groups. Adjustment for mode of feeding was performed since body composition is associated with method of infant feeding (Gale et al., 2012).

At 1 year, preterm infants had lower triceps skin fold thickness standard deviation score (1.4 ± 0.2) than term infants (2.1 ± 0.2). The mean difference, 0.8, 95%CI [0.3, 1.3], was significant $t = 3.3$, $p = 0.001$. There were no differences in subscapular skin fold thickness. (Figure 3.4)

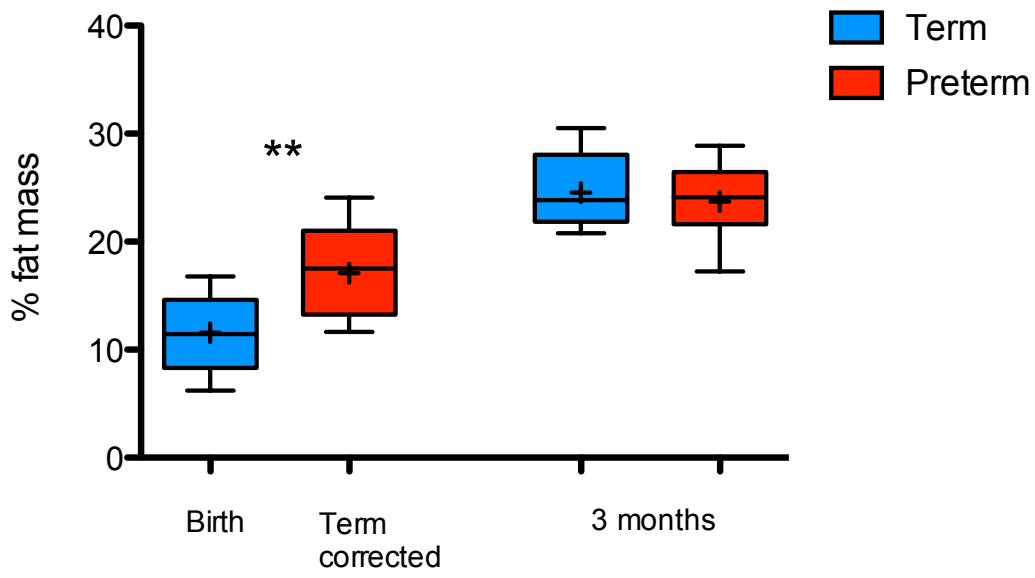


Figure 3.3 Percentage fat mass in term and preterm infants

Box and whisker plot: 10th to the 90th percentile with the line at median (+ indicates the mean). Term infants: n = 32 at birth and 30 at 3 months. Preterm infants: n = 21 at term corrected age and 32 at 3 months. **$p < 0.001$

		β [95% CI]	*t*	*p*
Model	Prematurity	-0.3 [-2.8, 2.2]	-0.2	0.82
	Male	2.1 [-4.2, -0.05]	-2.1	0.045
	Breast milk at 3 months	-0.3 [-2.7, 2.0]	-0.3	0.78

R^2 = 0.1 (p = 0.15)

Table 3.3 Multiple regression model of predictors of percentage fat mass at 3 months in preterm and term infants

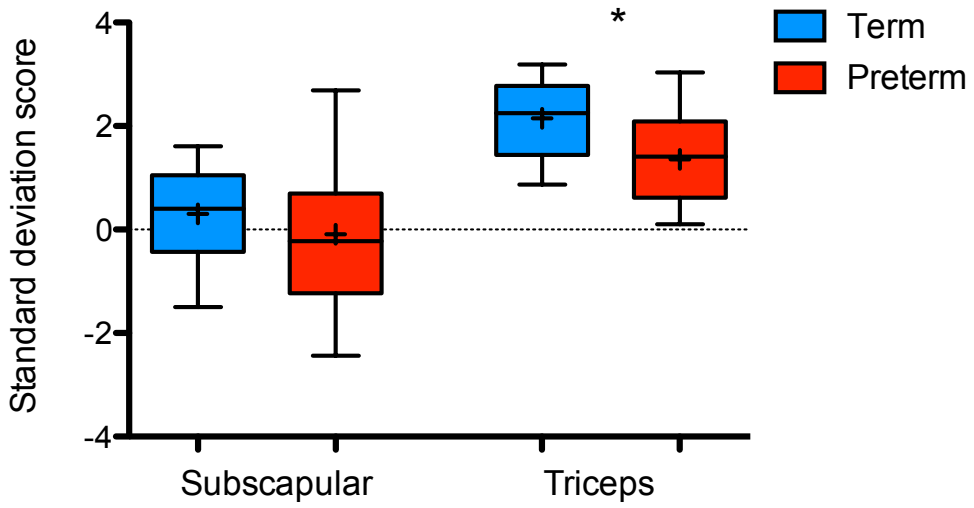


Figure 3.4 Skinfold thickness in term and preterm infants at 1 year

Box and whisker plot: 10th to the 90th percentile with the line at median (+ indicates the mean). Term infants $n = 32$. Preterm infants, $n = 31$. *$p < 0.05$

3.4.3 Salivary cortisol

Preterm infants demonstrated a blunted cortisol response to a stressor (0.23 ± 0.04 µg/dl) compared to term infants (0.31 ± 0.04 µg/dl) at 3 months. The mean difference, 0.4, 95%CI [0.05, 0.7], was significant, $t = 2.3$, $p = 0.02$.

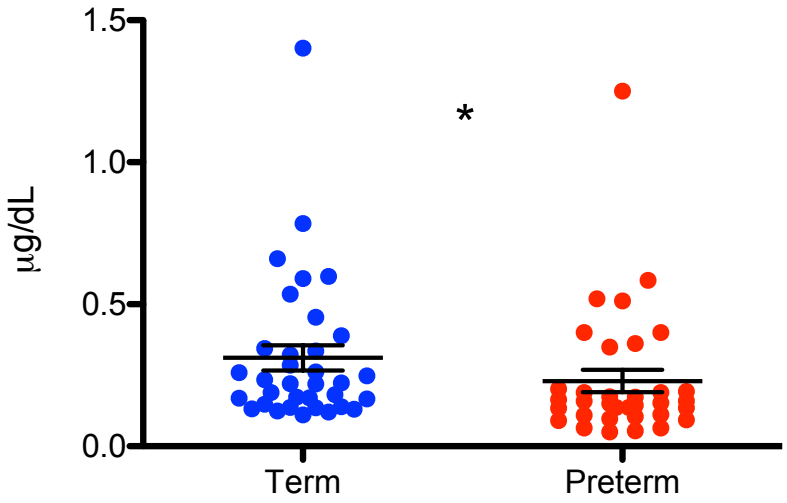


Figure 3.5 Salivary cortisol in term and preterm infants at 3 months

Scatter plot with mean ± SEM. $n = 34$ per group. *$p < 0.05$

3.4.4 DNA Methylation at *IGF2/H19*

3.4.4.1 *IGF2 DMR2*

Percentage methylation in term infants at birth at *DMR2* was 38.5 ± 0.8% and at 1 year was 40.3 ± 1.3%. The mean difference, -1.9 95%CI [-5.2, 1.4], was not significant $t = -1.3$, $p = 0.24$ (14 paired values).

Percentage methylation in preterm infants at birth was 28.3 ± 1.7%. This was markedly lower than in term infants at birth, mean difference = -10.2, 95% CI [-13.9, -6.5], $p < 0.001$. This remained significant in adjusted analysis, β = -11.5, 95%CI [-15.3, -7.7], $p < 0.001$. Social deprivation was an independent contributor towards reducing 5mC, β = -1.7, 95%CI [-2.9, -0.5], $p = 0.006$. None of the other covariates were significant contributors individually.

Percentage methylation in preterm infants at term corrected age was 36.6 ± 0.5%. This was significantly lower than in term infants at birth, mean difference = -2.2, 95%CI [-4.2, -0.2], p = 0.03. This remained significant in adjusted analysis, β = -2.8, 95%CI [-5.1, -0.6], p = 0.01. The significance was abolished when social deprivation was added into the model, β = -2.2, 95%CI [-4.3, 0.03], p = 0.053. Again, social deprivation appeared to be an independent contributor towards reducing methylation, β = -0.9, 95%CI [-1.6, -0.2], p = 0.02. None of the other covariates were significant contributors individually. The regression coefficients for all covariates are shown in Table 3.4.

	Predictors	β [95% CI]	*t*	*p*
Model 1	Prematurity	-2.2 [-4.2, -0.2]	-2.2	0.03
Model 2	Prematurity	-2.8 [-5.1, -0.6]	-2.6	0.01
	Male	2.0 [-0.1, 4.1]	1.9	0.07
	Maternal smoking	0.1 [-1.0, 1.2]	0.1	0.90
	Labour	0.2 [-1.8, 2.2]	0.2	0.83
Model 3	Prematurity	-2.2 [-4.3, 0.03]	-2.0	0.05
	Male	1.8 [-0.2, 3.8]	1.8	0.08
	Maternal smoking	0.02 [-1.0, 1.0]	0.03	0.97
	Labour	0.4 [-1.5, 2.4]	0.4	0.66
	Social deprivation	-0.9 [-1.6, -0.2]	-2.5	0.02

R^2 = 0.1 for model 2 (p = 0.33) and R^2 = 0.2 for model 3 (p = 0.02)

Table 3.4 Multiple regression models for predictors of percentage methylation at *IGF2 DMR2* in preterm infants at term corrected age and term infants at birth

The difference in methylation between preterm infants at birth and at term corrected age, -7.4, 95%CI [-12.5, -2.2], was significant t = -3.1, p = 0.01. However, the paired values available for analysis was small, n = 12.

At 1 year, percentage methylation in the preterm infants was 38.4 ± 0.7% and this was not significantly different from term infants, mean difference = -0.3, 95%CI [-

4.0, 3.3], p = 0.86. The effect of socioeconomic deprivation to reduce methylation approached significance, β = -0.9, 95%CI [-1.9, 0.1], p = 0.07. None of the other covariates were significant contributors individually.

The difference in mean methylation between preterm at term age and preterm at 1 year, -1.7, 95%CI [-3.8, 0.4], was not significant t = -1.7, p = 0.1 (21 paired values).

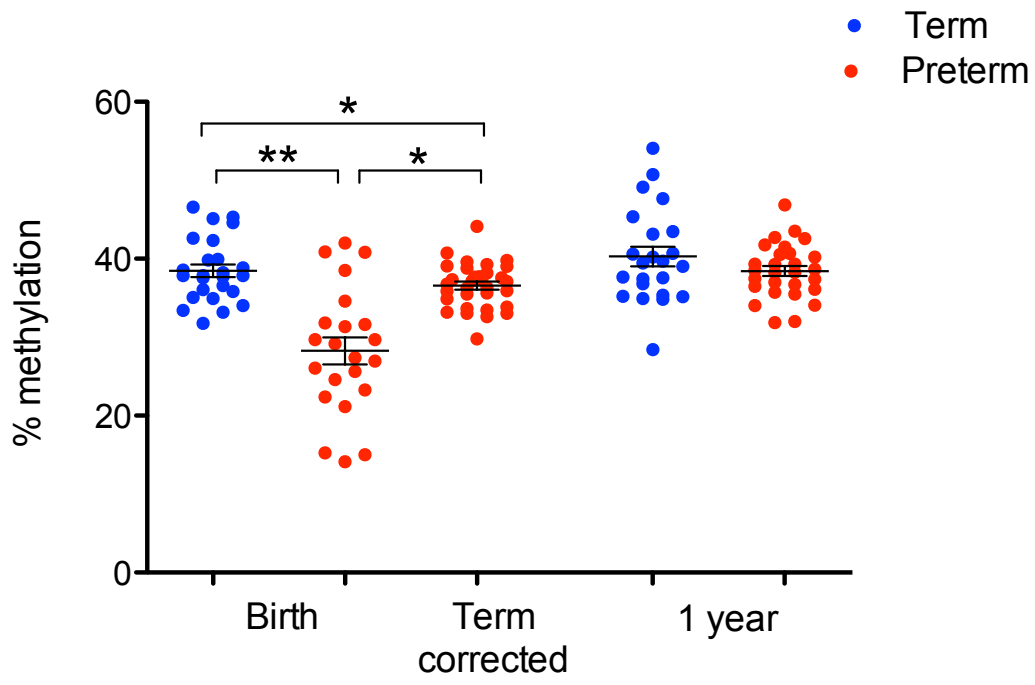


Figure 3.6 Percentage methylation at *IGF2 DMR2* in term and preterm infants

Scatter plot with mean ± SEM. Term infants: n = 25 at birth and 23 at 1 year. Preterm infants: n = 22 at birth, 32 at term corrected and 28 at 1 year. *p < 0.05 **p < 0.001

In univariate analyses, there was no relationship between percentage methylation at *IGF2 DMR2* and weight SD score at birth, R = 0.01, p = 0.98 or at 1 year, R = -0.08, p = 0.72 in the term infants.

In the preterm infants, birth weight SD score correlated strongly with percentage methylation at *IGF2 DMR2* at birth, $R = 0.71$, $p = 0$. There was no relationship between percentage methylation at this region and weight SD score at term corrected age, $R = 0.01$, $p = 0.96$, or at 1 year, $R = -0.06$, $p = 0.75$.

There was no relationship between percentage body fat and percentage methylation in term infants at birth, $R = 0.02$, $p = 0.94$, or in preterm infants at term corrected age, $R = -0.17$, $p = 0.51$.

The number of antenatal steroids in the preterm infants did not relate to percentage methylation at birth, Spearman's rho = -0.35, $p = 0.11$. The number of days of parenteral nutrition did not relate to percentage methylation at term corrected age, $R = -0.19$, $p = 0.15$.

3.4.4.2 *H19 ICR*

Percentage methylation at the *H19 ICR* in the term infants at birth was 49.2 ± 0.8% and was lower by 1 year, 45.4 ± 0.5%. The mean difference, 3.9%, 95%CI [1.4, 6.4] was significant, $t = 3.3$, $p = 0.004$ (22 paired values).

Percentage methylation at the *H19 ICR* in the preterm infants at birth was 48.1 ± 0.7%. The difference between term infants at birth, mean difference = -1.0, 95%CI [-3.2, 1.2], was not significant, $p = 0.35$. This remained non-significant in adjusted analysis, $\beta = -1.3$, 95%CI [-3.9, 1.2], $p = 0.3$. None of the covariates were significant contributors individually.

At term corrected age, percentage methylation in the preterm infants was 46.8 ± 0.5% and the difference with term infants at birth was significant, mean difference = -2.4, 95%CI [-4.4, -0.4], $p = 0.02$. This remained significant in adjusted analysis, $\beta = -2.3$, 95%CI [-4.6, -0.02], $p = 0.048$. None of the covariates were significant contributors individually. The difference in methylation between preterm infants at birth and at term corrected age, 1.9, 95%CI [-0.2, 4.0], was not significant $t = 2.0$, $p = 0.07$. However, the paired values available for analysis was small, $n = 13$.

At 1 year, percentage methylation in the preterm infants was 46.0 ± 0.6%. There was no significant difference with term infants at 1 year, mean difference = 0.6, 95%CI [-1.0, 2.3], p = 0.47. This remained non-significant in adjusted analysis, β = 0.8, 95%CI [-1.5, 3.0], p = 0.50. None of the covariates were significant contributors individually.

There was no significant reduction in percentage methylation observed in preterm infants at 1 year compared with at birth (mean difference = 2.6, 95%CI [-0.4, 5.7], t = 1.9, p = 0.09, (12 paired values)) or term corrected age (mean difference = 0.4, 95%CI [-1.4, 2.3], t = 0.5, p = 0.65, (20 paired values)).

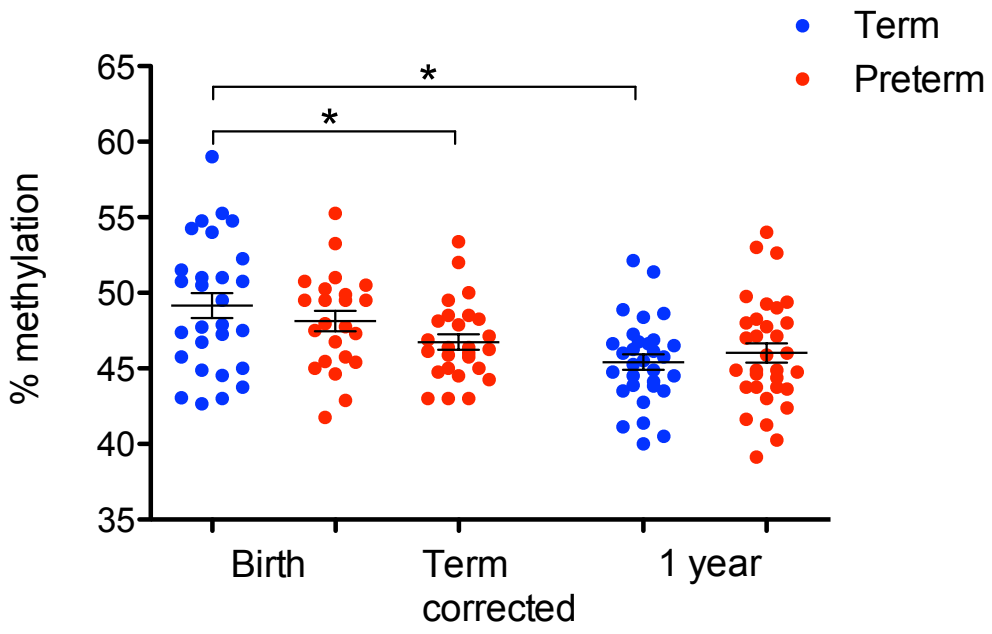


Figure 3.7 Percentage methylation at *H19 ICR* in term and preterm infants

Scatter plot with mean ± SEM. Term infants: at birth, n = 28 at birth, 31 at 1 year. Preterm infants: n = 23 at birth, 31 at term corrected, 32 at 1 year. *p < 0.05

In univariate analyses, there was no relationship between percentage methylation at *H19 ICR* and weight SD score at birth, R = - 0.15, p = 0.44 or at 1 year, R = 0.2, p = 0.28 in the term infants.

In the preterm infants, there was no relationship between percentage methylation at this region and weight SD score at birth, R = 0.18, p = 0.41, term corrected age, R = 0.18, p = 0.37, or at 1 year, R = 0.27, p = 0.14.

There was no relationship between percentage body fat and percentage methylation in term infants at birth, R = -0.22, p = 0.29, or in preterm infants at term corrected age, R = -0.16, p = 0.62.

The number of antenatal steroids in the preterm infants did not relate to percentage methylation at birth, Spearman's rho = -0.23, p = 0.29. The number of days of parenteral nutrition did not relate to percentage methylation at term corrected age, R = -0.01, p =0.95.

3.5 Discussion

In this study I established a cohort of preterm and term infants, obtained longitudinal data on anthropometry, measured salivary cortisol and related this to DNA methylation at *IGF2/H19*.

The preterm infants in this cohort were lighter at birth than the average preterm infant. This is over and above the fact that the reference SD scores are based on delivered preterm infants who are smaller than the healthy fetus at the same gestation (Bukowski et al., 2001) (Lackman et al., 2001). By term corrected age, preterm infants showed a downward deviation in weight SD score and were smaller than term infants. This pattern of growth during neonatal care is not dissimilar to that recently described for a population of infants born < 32 weeks gestation in the UK where following a drop in weight percentile, a steady state of growth is achieved, between

the 0.4th and 9th percentiles (Cole et al., 2014), albeit lower than in my cohort (mean weight percentile at term corrected age was 17.6 ± 3.3).

Additionally, at term corrected age, the preterm infants showed asymmetry with relative sparing of head growth. Growth restriction during the third trimester occurs as a result of factors such as placental insufficiency or maternal illness and there is a relative sparing of head growth (Kramer et al., 1989). Data suggests that this pattern of growth tends to persist in the neonatal unit, with spared head growth (Cockerill et al., 2006) resulting in asymmetric growth restriction at term corrected age (Uthaya et al., 2005). The data suggest that the preterm infants did not follow the expected fetal growth pattern, however it is not known which growth pattern in the neonatal period is ideal in the long-term with respect to metabolic health and intact neurodevelopmental health.

Following discharge from hospital and until 1 year, the preterm infants remained, on the whole, smaller than their full-term counterparts. However, the differences observed later in infancy were mainly as the term infants were larger than the reference mean. Although this gives the suggestion of catch-up growth, there was no statistically significant difference in rate of gain in body weight to support the contention that catch-up growth occurred in infancy. The infants may yet go on to do so in childhood and adolescence as described (Hack et al., 2003) which has been linked to later metabolic sequelae (Rotteveel et al., 2008) (Fewtrell et al., 2000). Nevertheless, the pattern of growth in infancy in this preterm cohort is typical of what has been published (Hack et al., 2003) (Roze et al., 2012). However, there are known to be sex differences in the pattern of growth in preterm infants: VLBW females show a greater increase in z-score (SD score) than males during infancy and also childhood (Hack et al., 2003). As the majority of my cohort was male, this may be another reason why I did not see increased growth velocity in my study.

Preterm infants had increased percentage fat mass as measured by PEAPOD at term corrected age but there was no persisting difference at 3 months; this is in keeping with previous independent studies using the same device (Roggero et al., 2009) (Carberry et al., 2010) (Ramel et al., 2011) (Simon et al., 2013). The measured

percentage fat mass values for the preterm and term infants were within the range of those published in these studies. Only 21 out of 44 preterm infants had PEAPOD assessment due to early discharge or complications of prematurity. These issues may have been faced by the independent research groups as well and so the measured values may not be accurate of all preterm infants reaching term corrected age. Complications such as BPD and dependence on parenteral nutrition are the severest on the neonatal unit and inability to include them may underestimate percentage fat mass (Uthaya et al., 2005).

The increased percentage fat mass (inferring a reduction in lean/fat-free mass as the preterm infants were smaller) at term corrected age probably reflects, at least in part, current nutritional practices during neonatal care. Due to difficulties in providing adequate intake to meet metabolic demands, preterm infants develop a cumulative protein and energy deficit that starts during the first weeks after birth (Embleton et al., 2001). Early growth and nutrition was shown to predict fat-free mass in preterm infants (Simon et al., 2014) particularly protein intake (Roggero et al., 2012) (Roggero et al., 2008). After term corrected age, the pattern suggests that preterm infants acquire relatively more fat-free mass and possibly indicates growth of various organs such as the brain. This increase in fat-free mass may be attributed to formula feeding that was predominant in the preterm cohort and this phenomenon has been described in a systematic review (Gale et al., 2012). Whether the use of air displacement plethysmography would detect changes in body composition beyond 6 months is unknown as this is not practical using the PEAPOD device. This would be important to know as the highest change of fat mass in relation to body weight is in infancy and not limited to the first 6 months (Fomon et al., 1982). Nevertheless, air displacement plethysmography does not describe the distribution of body fat, for which MRI would be useful. Two different studies have shown that preterm infants at term corrected age and adults born preterm have more visceral fat and less subcutaneous fat compared with those born at term (Uthaya et al., 2005) (Thomas et al., 2011). The reduced triceps skin fold thickness at 1 year in my study could mirror this feature. However, we did not measure skin fold thickness earlier in life to corroborate this.

Preterm infants had lower salivary cortisol levels than term infants at 3 months. The measurements were all done in the afternoon at the end of the clinic visit during which the infants were undressed and had growth measurements performed including the body composition measurement using the PEAPOD. The blunted response in the preterm group to this activity may be attributed to suppression from antenatal steroids as proposed in the literature (Tegethoff et al., 2009) (Glover et al., 2005) as 96% of preterm infants in this cohort were exposed to at least one dose of dexamethasone. Adding to this would be endogenous cortisol release whilst being exposed to stressful conditions during intensive care (Ng et al., 2011) (Glover et al., 2005) and possibly during fetal life. The difference observed is despite that most of the term infants were born by elective Caesarean section which may associate with an altered HPA axis activity and a lower cortisol response to a stressor in early infancy (Taylor et al., 2000) (Miller et al., 2005). Following lower cortisol levels in early infancy, the trajectory is for higher levels in late infancy and toddlerhood (Grunau et al., 2007) and altered cortisol homeostasis may have collateral effects on developing organ systems that are long lasting. An altered HPA axis may persist into adulthood, which has been associated with a myriad of cardiovascular risk factors including central adiposity (Reynolds, 2013a). The low basal cortisol levels may have promoted the acquisition of lean mass by 3 months following reportedly high levels of cortisol during neonatal intensive care (Ng et al., 2011) (Glover et al., 2005) which is thought to promote the central adiposity at term corrected age (Uthaya et al., 2005). It would be informative to evaluate this cohort later in childhood and adolescence to see whether the altered basal cortisol levels manifest into an altered HPA axis and whether this has bearing on body composition and precursors of cardiovascular or metabolic disease such as elevated BP or insulin resistance.

Preterm infants had lower percentage DNA methylation at both *H19 ICR* and *IGF2 DMR2* than term infants early in life. The *H19 ICR* is methylated on the paternal allele, which prevents binding of CTCF and therefore allows the enhancers to promote *IGF2* expression. Similarly, *IGF2 DMR2* is methylated on the paternal allele and promotes expression of *IGF2* (Figure 1.2) (Phillips & Corces, 2009) (Kurukuti et al., 2006). In theory, the reduced DNA methylation at *H19 ICR* and

IGF2 DMR2 in the preterm infants at term corrected age would be predicted to reduce *IGF2* expression. This could be one explanation for the observed growth faltering at term corrected age and that there were no persistent differences in DNA methylation at 1 year when the growth parameters were not as discrepant. Should altered DNA methylation at *IGF2* have a bearing on growth, it must be noted that the absolute differences in DNA methylation at term corrected age are small and may be overestimated as the term cohort had greater SD scores than the reference mean. On the other hand, there were no statistically significant relationships between weight SD score and DNA methylation at any time point other than with *IGF2 DMR2* at birth in preterm infants.. This points towards IGF2 being the major prenatal growth factor and whilst recent research suggests that it has a role in postnatal growth (Begermann et al., 2015) it is not known what the relationship is between circulating IGF2 and DNA methylation at 1 year. The lack of difference at 1 year is similar to the findings of a genome wide study of methylation from blood in preterm and term subjects at birth and then at 18 years in which differences in DNA methylation seen at birth were not persistent at 18 years (Cruickshank et al., 2013). The reduction in methylation at the *H19 ICR* between birth and 1 year may be attributed to the gradual loss of methylation that has been described to occur with time (Fraga et al., 2005) (Heyn et al., 2012).

The changes in DNA methylation at *IGF2 DMR2* are particularly interesting. Results from a mouse study suggest that DNA methylation at this DMR is particularly susceptible to nutritional modification whilst methylation at the other DMRs regulating *IGF2* allelic expression are more stable (Waterland, 2006). To support this, the results of a separate study of *IGF2/H19* in human newborn tissues showed the greatest and least variation in methylation at sites corresponding to *IGF2 DMR2* and *H19 ICR* respectively (Ollikainen et al., 2010). Additionally, in contrast to the other DMRs, *IGF2 DMR2* demonstrates developmental changes throughout the postnatal period and into adulthood with a gradual increase in DNA methylation over time (Waterland, 2006). We observed stark differences in DNA methylation at *IGF2 DMR2* between preterm infants at birth and term infants at birth and a lesser difference at term corrected age. We can postulate based on this, that methylation at

IGF2 DMR2 also undergoes developmental changes in humans, at least during late gestation. The persistent reduction in methylation at term corrected age may reflect the altered nutritional state of the infants born preterm, but may be overestimated due to the term infants being larger than expected. We did not see any relationship between indicators of altered nutrition such as length of dependence on parenteral nutrition or percentage body fat at term corrected age and DNA methylation at *IGF2 DMR2*. But, simple univariate analyses may not capture any relationship, should it exist, between many determinants of nutritional state and DNA methylation.

Social deprivation as quantified by DEPCAT score was an independent predictor of reduced methylation at *IGF2 DMR2* in early life with a trend towards this at 1 year. Social deprivation appeared to have a greater effect than prematurity on reducing methylation at term corrected age than at birth. Inter-related factors such as smoking (Elliott et al., 2014) and malnutrition (Heijmans et al., 2008) that contribute to stressors associated with socio-economic deprivation can impact on DNA methylation. Indeed a recent study revealed a relationship between childhood socioeconomic status and adult DNA methylation (Borghol et al., 2012), which supports our findings.

DNA methylation at *IGF2 DMR2* appeared to be more susceptible to early life events (factors associated with social deprivation and preterm birth) than *H19 ICR*. The variation that we observed supports the notion that imprinting is established at primary regions (e.g. *H19 ICR*) during gametogenesis and these marks are generally maintained during development (Edwards & Ferguson-Smith, 2007). Imprinting is established at secondary regions (e.g. *IGF2 DMR2*) as a consequence of its establishment at primary regions following fertilisation (Edwards & Ferguson-Smith, 2007). Hence, these secondary regions may be more vulnerable to environmental influences, such as stress during gestation and the postnatal period or preterm birth, than primary regions. This is nevertheless an observational study and causation cannot be assumed. Whilst groups were mixed to reduce batch effect, I was not blinded to study groups and bias may have been introduced.

We only examined DNA methylation at two DMRs (*IGF2 DMR2* and *H19 ICR*) and only at a short sequence of CpG dinucleotides within these loci. Study of the remaining regions as well as the study of *IGF2 DMR0* is desirable. This was a candidate gene approach and although genome wide analyses have been done in other studies, the sample sizes were smaller (Parets et al., 2013) (Cruickshank et al., 2013). Further study with an unbiased method may reveal additional interesting findings in other loci, but this is very costly for cohort studies with average to large sample sizes. Twins were included in this study as they are over-represented in preterm populations. This poses questions as to how twins are treated in study designs and analyses as their measured values do not meet the assumption of independence due to shared genetic and environmental factors. Twins were only in the preterm group and we chose to analyse them individually because we were interested in comparing preterm infants as a group with term infants. Whilst there were 13 preterm infants borne out of twin pregnancy, there were only 5 complete twin pairs. So the impact may be small and methods to account for correlational nature of the measurements may fail with small samples and classical statistical techniques may be sufficient (Shaffer et al., 2009). However, it may be more important to take into account twin pairs in regression modelling involving only preterm infants. Measuring salivary cortisol at birth / term corrected age was not possible as babies at this age do not salivate enough to collect a sufficient volume using sorbettes (BD Visitec, Massachusetts, USA). Salimetrics latterly manufactured a variety of swabs for different age groups allowing larger volumes to be collected. However infants at 1 year are unwilling to comply (and more able to resist) in keeping the swab in the mouth for the required duration, particularly following collection of saliva using 5 sponges for DNA which was prioritised. Older children may comply more enabling longitudinal cortisol analysis. When gathering measurements and collecting samples, I was not blinded to the gestation of the infant, thus unconscious bias may have factored.

Despite the limitations, this study has several strengths. This was a prospective study and there was far less than the 50% drop out anticipated. The measurements were taken by one observer the majority of the time. The PEAPOD is a validated tool for

estimating body composition and was calibrated prior to each use. Saliva for cortisol was collected at the end of the clinical examination and the follow up appointments at 3 months were consistently held in the afternoons for this purpose. In saliva, the majority of the cortisol remains unbound to protein whereas in the blood, only 1 to 15% of cortisol is in the unbound form. It is the unbound fraction that is biologically active and as there is report of high correlation between serum and saliva cortisol levels (Vining et al., 1983), we are confident that salivary cortisol measurements reliably reflect HPA axis activity.

In summary, in this cohort of preterm infants who did not achieve the reference for growth by the time they reached 1 year of age, with lower basal cortisol levels in infancy, there were evolving changes in DNA methylation. These were at key regions of *IGF2/H19* from mid-gestation until 1 year and preterm birth associated with decreased DNA methylation in the early postnatal period with apparent normalising by 1 year. As such, DNA methylation marks at these regions are unlikely to afford stable biomarkers of risk. Further analysis of other key regions is important, as would be an unbiased analysis. Follow up of the cohort would be desirable, firstly, to determine whether suppression of cortisol remains; follow up at school age may be ideal as this may reveal subtle differences in behavior or school performance. Thereafter, follow up in adolescence may reveal changes to the epigenome that may accompany dramatic changes in growth brought on by puberty.

In addition to comparing how DNA methylation in preterm infants may differ from term infants, we aimed to study DNA methylation in relation to brain development. This is important, given that preterm birth poses a large burden of neurodevelopmental adversity in terms of special educational needs (MacKay et al., 2010) and later psychiatric illness (Nosarti et al., 2012). Achieving both maximal neurodevelopmental outcome and optimum metabolic status may be difficult. In the next chapter, I studied relationships between DNA methylation and brain MRI findings.

Chapter 4: DNA methylation and preterm brain development

IntroductionThe main neuropathology in the preterm infant comprises periventricular leucomalacia and neuronal/axonal disease. This accounts for the majority of the observed neurodevelopmental abnormalities and collectively, this is now known as "the encephalopathy of prematurity" (Volpe, 2009). The cognitive component is attributed mainly to neuronal/axonal disease (Volpe, 2009) and rather than being separate conditions, there is considerable clinical overlap between cognitive impairment and other developmental disorders such as autism (Johnson & Marlow, 2011). Schizophrenia is at the extreme end of the spectrum of disorders characterised by disturbance of cognition, affect and behaviour, but is commonly studied in adults as a hard end point and is considered to be a disease largely of white matter (Davis et al., 2003).

Conventional and advanced MRI tools have identified neonatal imaging phenotypes following preterm birth which include reduced cortical complexity (Woodward et al., 2006) tissue volume reduction (Boardman et al., 2010). Additionally, microstructural integrity of white matter tracts can be measured using diffusion MRI (dMRI) and tractography (Basser et al., 2000). The resulting metric, tract based fractional anisotropy (FA), can also indicate the reduced connectivity of neural systems that occur in preterm infants (Pandit et al., 2014). In addition to changes associated with preterm birth, other adverse early life environmental exposures such as maternal exposure to famine around the time of conception have been associated with structural brain abnormalities (focal white matter hyper-intensity and decreased brain volume) and an increased risk of psychiatric disease in the offspring (Hulshoff Pol et al., 2000).

Imprinted genes are recognised to play a key role in normal neurodevelopment and brain function (Wilkinson et al., 2007) and it has been proposed that disorders such as autism and schizophrenia may have their origin in alterations of the normal balance of imprinted gene expression (Badcock & Crespi, 2008). In particular, *IGF2* is expressed in the brain in a parent of origin specific manner, and has been shown to

have roles in brain function such as cognition and memory (Chen et al., 2011) and in synapse formation (Schmeisser et al., 2012). DNA methylation at the *IGF2*/*H19* locus in peripheral blood can be altered by early life stressors such as famine (Heijmans et al., 2008) and maternal malnutrition (Drake et al., 2012). It has been hypothesised that the association between DNA methylation at *IGF2* and adult brain weight may explain the epidemiological link between psychiatric disease and brain abnormalities in individuals exposed to pre-natal famine (Pidsley et al., 2009). Given that preterm birth may be associated with altered DNA methylation at *IGF2*/*H19*, these alterations may be one mechanism underpinning abnormal brain development after preterm birth.

4.1 Hypothesis

DNA methylation at the *IGF2/H19* locus relates to FA, a measure of microstructural integrity of key white matter tracts, and/or whole brain volume in preterm infants at term equivalent.

4.2 Methods

4.2.1 Subjects

Following ethical approval and written parental consent, preterm infants were recruited whilst on the neonatal unit. Demographic details and history were obtained from the infant and mother's case notes and during the study visit. Characteristics are shown in Tables 4.1 and 4.2.

The study sample consisted of 50 infants born at less than 1500g or less than 32 weeks' gestation. Exclusion criteria were: major congenital abnormality, post-haemorrhagic ventricular dilatation or porencephalic cysts.

4.2.2 Laboratory methods

Saliva for buccal DNA was collected at the time of MRI scan acquisition at term corrected age (mean PMA 39.8, range 37+6 to 42+4). Saliva was collected using Oragene DNA (OG-250) kits. DNA was extracted following the manufacturer's guidance. Percentage methylation was analysed using Pyrosequencing after bisulphite conversion.

4.2.3 MR Image acquisition and analysis

A Siemens Magnetom Verio 3 T MRI clinical scanner (Siemens AG, Healthcare Sector, Erlangen, Germany) and 12-channel phased-array head coil were used. White matter tract integrity was measured by probabilistic neighbourhood tractography (Anblagan et al., 2015). dMRI images were pre-processed using FSL (FMRIB, Oxford, UK; http://www.fmrib.ox.ac.uk). FA for every infant was generated using "DTIFIT". Tracts of interest (TOI) were: genu and splenium of corpus callosum, cingulate cingulum gyrus, corticospinal tract and inferior longitudinal fasciculus. Whole brain volume data were computed according to published methods (Serag et al., 2012).

Gestation at birth, weeks	29.2 (23+2 – 33+0)
Birth weight, g	1165 (586 – 1635)
Birth weight SDS	-0.56 (-2.61-0.95)
PMA at image acquisition, weeks	39.8 (37+6 -42+4)
Weight SDS at image acquisition	-1.35 (-3.34 – 0.36)
OFC SDS at image acquisition	0.01 (-2.86 – 2.55)
Male, *n* (%)	29 (58)
Bronchopulmonary dysplasia, *n* (%)	15 (30)
Laser for retinopathy of prematurity, *n* (%)	1 (2)
Necrotising enterocolitis, *n* (%)	2 (4)
Intraventricular haemorrhage, *n* (%)	2 (4)
Periventricular leucomalacia, *n* (%)	2 (4)
Late onset sepsis, *n* (%)	22 (44)

Table 4.1 Characteristics of the study participants

Values are means (range). $N = 50$ infants. Bronchopulmonary dysplasia is defined as need for respiratory support and/or supplemental oxygen at 36 weeks' PMA to maintain oxygen saturations of 90% or more. Necrotising enterocolitis is defined as Bell stage 2 or greater. Intraventricular haemorrhage is defined as only grade III or IV events. The definition of late onset sepsis is taken from the Vermont Oxford Network Manual of Operations, release 16.3. Abbreviations: PMA – postmenstrual age, SDS – standard deviation score, OFC – occipito-frontal circumference

Age, years	30.4 (17 – 40)
BMI, kg/m^2	24.8 (18 – 36)
DEPCAT score, mode	3
Caucasian ethnicity, *n* (%)	41 (95.3)
Smoking, *n* (%)	
Current	10 (23.3)
Former	15 (34.9)
Never	18 (41.9)
Primiparity, *n* (%)	29 (67.4)
Folic acid during first trimester, *n* (%)	41 (95.3)
Assisted reproduction, *n* (%)	5 (11.6)
Multiple pregnancy, *n* (%)	14 (32.6)
Antenatal steroids, *n* (%)	
Any exposure	40 (93.0)
Complete course	29 (67.4)
Antenatal Magnesium sulphate, *n* (%)	20 (46.5)
Caesarean section, *n* (%)	31 (72.1)

Table 4.2 Maternal characteristics of the study participants

Values are means (range). N = 43 mothers. A complete course of steroids was defined as the first dose of dexamethasone administered more than 24 hours before delivery. Abbreviation: DEPCAT – deprivation category.

4.2.4 Statistics

Since in general, neighbouring CpG sites tend to have similar levels of methylation (Nautiyal et al., 2010), percentage DNA methylation is expressed as the average across all CpG sites in the locus tested rather than at individual CpG sites and analysed as such.

Pearson's correlation coefficients were measured between percentage methylation (at *H19 ICR* and *IGF2 DMR2*), whole brain volume, FA at genu and splenium of corpus callosum, cingulate cingulum gyrus, corticospinal tract and inferior longitudinal fasciculus.

In a linear regression model, whole brain volume and FA for each white matter tract were dependent (outcome) variables and age at image acquisition was added as an

independent variable. Age influences both whole brain volume and FA (Pandit et al., 2014). The standardised regression (β) coefficients from these models indicate the number of standard deviations that whole brain volume / FA will change as a result of one standard deviation change in the other predictors. Data is expressed as mean ± SEM and standard deviation in brackets. Values were normally distributed. Statistical significance was set at 0.05 (2 tailed). Dr James Boardman performed the statistical analysis using SPSS v21 (SPSS Inc, Chicago, USA).

4.3 Results

Percentage methylation at the *H19 ICR* was 48.6 ± 0.5% (SD = 2.8) and at *IGF2 DMR2* was 38.0 ± 0.8 % (SD = 5.0). Whole brain volume was 450 ± 6.9 cc (SD = 48.4).

	Mean	SEM	SD
Genu CC	0.22	0.006	0.04
Splenium CC	0.28	0.007	0.05
CST	0.29	0.005	0.04
CCG	0.21	0.005	0.03
ILF	0.22	0.005	0.03

Table 4.3 FA values for the main white matter tracts

Abbreviations: SEM – standard error of the mean, SD – standard deviation, CC – corpus callosum, CST – corticospinal tract, CCG – cingulum cingulate gyrus, ILF – inferior longitudinal fasciculi

There was no significant correlation between percentage methylation at either site and whole brain volume. Nor were there any significant correlations between percentage at either site and FA at each of the major white matter tracts.

For *H19 ICR*, age at image acquisition was a significant predictor in the model for FA in the genu of the corpus callosum (standardised $\beta = 0.38$, $p = 0.04$), corticospinal tract (standardised $\beta = 0.74$, $p < 0.001$) and the cingulum cingulate gyrus (standardised $\beta = 0.54$, $p = 0.01$).

For *DMR2*, age at image acquisition was a significant predictor in the model for whole brain volume (standardised β = 0.36, $p = 0.046$), genu of the corpus callosum (standardised β = 0.43, $p = 0.02$), corticospinal tract (standardised β = 0.72, $p < 0.001$) and the cingulum cingulate gyrus (standardised β = 0.56, $p = 0.003$).

Percentage methylation at either locus was not significant in any model where FA was the dependent variable. Nor was percentage methylation at either locus a significant predictor of brain volume (Table 4.4).

		r	*p*	Standardised β	*p*
H19 ICR	Whole brain volume	-0.10	0.58	-0.02	0.92
	Genu CC FA	0.04	0.83	0.14	0.43
	Splenium CC FA	-0.03	0.90	-0.01	0.95
	CST FA	0.01	0.95	0.21	0.12
	CCG FA	-0.01	0.96	0.10	0.58
	ILF FA	-0.12	0.50	-0.05	0.79
DMR2	Whole brain volume	-0.04	0.84	-0.14	0.94
	Genu CC FA	-0.18	0.34	-0.12	0.48
	Splenium CC FA	0.29	0.11	-0.08	0.68
	CST FA	-0.09	0.63	-0.04	0.75
	CCG FA	-0.03	0.90	0.07	0.68
	ILF FA	-0.07	0.72	-0.11	0.52

Table 4.4 Relationships between DNA methylation and regions of the preterm brain

Abbreviations: CC – corpus callosum, CST – corticospinal tract, CCG – cingulum cingulate gyrus, ILF – inferior longitudinal fasciculi

4.4 Discussion

The results of this study do not support a relationship between DNA methylation at the *IGF2/H19* locus in peripheral cells and white matter microstructure or whole brain volume as measured by MR.

White matter (preterm brain) abnormality results from a combination of destructive processes (free radicals, ischaemia, inflammation, excitotoxicity) and maturational

processes (Volpe, 2009) during the antenatal and neonatal period. We postulated that DNA methylation at *IGF2 DMR2* and *H19 ICR* might mediate early life stressors on processes such as inflammation and maturation. Microstructural integrity of white matter at term equivalent age was measured by dMRI and the resulting metric used, FA, gives a measure of axonal size, pre and true myelination, fibre density and coherence. Therefore, whilst it is very sensitive, it is non-specific (Nossin-Manor et al., 2013). Additionally, the tract averaged FA values give an indication of the strength of connectivity in the tracts (Pandit et al., 2014). The results in this study suggest that DNA methylation at *IGF2/H19* may not be associated with these processes.

This is important given that a tract such as the splenium of the corpus callosum is involved in relevant neurodevelopmental outcomes. Volume loss in the splenium of corpus callosum has been described in ADHD (Valera et al., 2007) and a meta-analysis concluded that there is reduced FA in patients with schizophrenia (Patel et al., 2011). As a whole, the corpus callosum is smaller in individuals with autism (Stanfield et al., 2008) and in children born preterm, the size (Nosarti et al., 2004) and FA (Counsell et al., 2008) of the corpus callosum relates to measures of cognition.

Data on the relationship between DNA methylation at *IGF2/H19* and brain size are limited to a few studies in adults. DNA methylation at *IGF2 DMR2* in adult cerebellar tissue correlated in a positive manner with total brain weight, but only in males (Pidsley et al., 2009). In contrast, a negative correlation between DNA methylation at the CTCF binding sites (*H19 ICR*) in cerebellar tissue and cerebellar weight in humans (Pidsley et al., 2012a) and mice (Pidsley et al., 2012b) was observed. No relationship was observed with net brain mass in either of the latter two studies. However, DNA methylation at *H19 ICR* should, in theory, block the binding of the zinc-finger protein CCCTC-binding factor (CTCF) and the assembly of a chromatin insulator. This should then allow functional communication between the promoter of *IGF2* and the enhancers and activate *IGF2* expression (Phillips & Corces, 2009) (Kurukuti et al., 2006). Thus, we should expect a positive relationship between DNA methylation at the *H19 ICR* and cerebellar weight. DNA methylation

levels at *IG2/H19,* particularly *H19 CTCF3,* is reported to be similar between 4 different brain areas, peripheral blood and buccal cells with mean methylation of 40% (SD < 0.01) (Pidsley et al., 2012a). The differences between studies and the lack of a positive relationship may reflect the presence of 5-hydroxymethylcytosine in the brain (Kriaucionis & Heintz, 2009) that is not accounted for during bisulphite conversion and Pyrosequencing (Huang et al., 2010).

Adolescents born preterm have been demonstrated to have smaller brain volumes (Nosarti et al., 2002) but, this was not found to be the case at term equivalent age in a select group of preterm infants without major parenchymal injury cared for in a modern neonatal unit (Boardman et al., 2007). Our cohort may have similar relative preservation of brain volume. Although not specifically measured, our sample of infants may not have had cerebellar volume loss either. This may be relevant as cerebellar growth appears to be conserved in preterm infants at term equivalent age in the absence of supra-tentorial lesions (Srinivasan et al., 2006) and infants with such lesions would have been excluded from imaging in our study.

Nevertheless, initial normal cerebellar volumes may be followed by diminished later growth in association with cognitive deficits in individuals born preterm (Allin et al., 2001). The cerebellum undergoes a lengthy period of development where it is last of the brain structures to reach maturity (Tiemeier et al., 2010) and it is a region important for cognition and behaviour (Schmahmann, 2004). This is borne out further in clinical studies: in autism, the cerebellar vermal lobules were found to be reduced in size with a different developmental trajectory (Stanfield et al., 2008) and in ADHD (Valera et al., 2007). The developmental trajectories of the cerebellum are sexually dimorphic and this may be the reason males are predisposed to these psychiatric disorders (Tiemeier et al., 2010). Thus, future studies could interrogate the relationship between DNA methylation at *IGF2/H19* and cerebellar structure in children or adolescents born preterm.

A further region of interest would be the hippocampus given the data suggesting that it is vulnerable to stress. Hippocampal volume measurements by MR were reduced in adult subjects experiencing major psychological stress such as combat related post-

traumatic stress disorder (Bremner et al., 1995), child abuse (Teicher et al., 2012) and major depression (Sheline et al., 1996). This is substantiated by experimental data showing reduced hippocampal neuron numbers and smaller MR based hippocampal volumes in primate offspring exposed to antenatal dexamethasone (Uno et al., 1994). Altered DNA methylation at *NR3C1* in the hippocampus has been shown in experimental models of early life stress (Weaver et al., 2004) and human studies (McGowan et al., 2009) and may mediate the abnormal stress reactivity seen in childhood. Volume decrease in the amygdala (where glucocorticoids have further action) has also been noted following early life stress and associated with behavioural difficulties in childhood (Hanson et al., 2015). MRI techniques to quantify hippocampal and amygdala volumes in early life would need to be optimised and these measures could be studied in relation to DNA methylation at *NR3C1*.

Prior specification of the required sample size for this study was not possible as there is no established method for power calculation in this type of study. Using an online tool, www.danielsroper.com for a post hoc power calculation using observed R^2 of 0.001 for the model with whole brain volume as dependent variable and DNA methylation at *DMR2* as an independent variable, the power was 0.89. So, as the study is powered to detect a relationship, we can be confident that there is not one. Nevertheless, we studied a sample of very preterm infants, and it is possible that a sample spanning from very preterm to full term may tease out a relationship between MR measurements and DNA methylation. As before, twins were included in the study as they form a large proportion of preterm infants. Whilst there were 21 preterm infants borne out of twin pregnancy, there were only 7 complete twin pairs. However, due to the possibility that both FA and percentage methylation values may have a correlational nature (from shared genetic and environmental factors), it may have been important to take into account twin pairs in regression modelling (Carlin et al., 2005). Including twin pairs alone may be more informative in studying epigenetic variation and brain structure given the high heritability of IQ and brain structure (Kohannim et al., 2012). Any discordance in DNA methylation in twins could be

attributed mostly to environmental (including the non-shared intrauterine) and stochastic factors whereas genetic contributions will be less (Gordon et al., 2012).

In this study, DNA methylation in buccal cells served as a proxy for gene function in the brain, but this is considered to be adequate as DNA methylation profiles of candidate genes and methylome wide in buccal cells are reportedly similar to those in the brain (Pidsley et al., 2012a) (Smith et al., 2014). Whilst the findings here showed no relationship, it cannot be excluded that there might be changes detectable in the brain matter. Post-mortem samples would then be necessary with DNA methylation finding compared with histo-pathological measures rather than MR imaging and extrapolation to childhood outcomes would not be valid. Further studies could address genetic variation and its relation to brain structure in prematurity. It would be important to do so given the high heritability of IQ and identification of common genetic variants that modify IQ (Caspi et al., 2007) but also relate to white matter integrity in adults (Kohannim et al., 2012) and preterm infants (Boardman et al., 2014). Of particular interest is the gene FK506 binding protein 5 (*FKBP5)* and its single nucleotide polymorphism (SNP) *rs1360780* that predisposes to affective disorders (Binder et al., 2008). *FKBP5* modulates the sensitivity of the glucocorticoid receptor and early life stress exposure in the presence of *rs1360780* leads to epigenetic change in the glucocorticoid response elements of *FKBP5* resulting in imbalance of *FKBP5* and *NR3C1* activity and in the long term, increasing the risk of psychiatric disease (Klengel et al., 2012). Additionally, the presence of *rs1360780* is associated with reduced FA in the posterior cingulum tract, a recognised imaging phenotype in depression and post-traumatic stress disorder (Fani et al., 2014). Similarly, the early life stress associated with preterm birth may interact with the presence of *rs1360780* to predispose to white matter injury and neuro-psychiatric disease. This would be interesting to study further.

In summary, our results do not suggest that DNA methylation at *IGF2/H19* locus modulates brain development in preterm infants as measured by MR. Further studies could investigate whether specifically cerebellar development in later life relates to DNA methylation at this locus. Ultimately, inherited traits play a major role and the

study of gene-environment interactions may yield further clues to early brain development.

Chapter 5: Telomere biology in programming

5.1 Introduction

We sought to utilise the cohort established and described in Chapter 3 to investigate an additional potential marker of early life stress – telomere attrition. This has received much research attention recently, yet very little is known about telomere biology in the perinatal period or in preterm infants.

Telomeres are strings of repetitive, non-coding DNA sequences that cap the end of chromosomes and preserve genomic DNA integrity. During mitosis, due to a limitation of the function of DNA polymerase, a short part of the telomeric DNA is not fully replicated leading to telomere shortening with every replication. Eventually, cells with enough depleted telomeres undergo senescence (Blackburn, 1991) and consequently, telomeres offer a marker of cellular ageing.

The association between short telomere length and mortality (Cawthon et al., 2003) and age-related illnesses such as cardiovascular disease (Samani et al., 2001) suggests that they can be markers of biological ageing and not just chronological ageing (Lindsey et al., 1991). The accumulating evidence that stress affects telomere integrity may be a mechanism linking stress and age-related illness. Psychological stress in pre-menopausal women has been shown to associate with shorter telomere length (Epel et al., 2004). In childhood, social deprivation associated with shorter telomeres (Mitchell et al., 2014) and exposure to violence associated with telomere attrition (Shalev et al., 2012). Intra-uterine stress was shown to associate with shorter telomere length in the offspring as adults (Entringer et al., 2011). This suggests that early life adversity may alter the trajectory of telomere attrition with measurable differences in telomere length in later life.

Whilst some studies suggest that adults (Entringer et al., 2011) and children (Raqib et al., 2007) born small have shorter telomeres, no differences in telomere length was observed in a large cohort of adults with low birth weight or very preterm birth (Kajantie et al., 2012). Of note, this was a cohort where birth status predicted a

number of adult diseases and their risk factors such as hypertension, type 2 diabetes or impaired glucose tolerance (Kajantie et al., 2012).

There are, however, very few studies on telomere biology in early life or in preterm infants. Whilst data do not indicate that fetal growth restriction associates with shorter telomeres at birth (Akkad et al., 2006), it appears that telomere length is inversely proportional to gestational age – at least when measured at a single time point in a range of preterm infants at birth (Friedrich et al., 2001) (Menon et al., 2012). Serial measurements during hospital stay indicated steady shortening of telomeres in preterm infants compared with unborn fetuses matched for gestational age (Holmes et al., 2009) and although this was a very small study (total $n = 13$), this suggests premature cellular ageing in the preterm group. Mechanisms proposed for this include oxidative stress (Kawanishi & Oikawa, 2004) (Zglinicki, 2002) or inflammation (Wong et al., 2014), both of which are known to cause telomere erosion and are implicated in morbidity in the neonatal period in preterm infants (Vento et al., 2012) (Dammann & Leviton, 2000). These previous studies were, however limited to the perinatal period, the preterm groups did not have telomere length measured at term corrected age and there was no longitudinal study in infancy or childhood.

As opposed to the mean telomere restriction fragment method used in the study by Holmes et al (Holmes et al., 2009) and Friedrich et al (Friedrich et al., 2001), the qPCR method (Cawthon, 2002) used by Menon et al (Menon et al., 2012) allows for larger numbers of samples to be analysed. Comparing telomere length measurements of a larger sample of preterm infants with term infants from birth over an equivalent time period is required to determine whether preterm infants have faster telomere attrition and to explore the possibility of telomere length as a marker of early life stress response.

5.2 Hypothesis

I aimed to test the hypothesis that preterm birth is associated with faster telomere attrition during the first year of life compared with term infants.

5.3 Methods

5.3.1 Subjects

The study sample consisted of 86 infants as described in Chapter 3: 40 infants born at full term and 46 born at less than 32 weeks gestation. Saliva for buccal DNA was collected within the first week after birth and at 1 year corrected age. For the preterm infants, additional saliva was collected at term corrected age.

5.3.2 Relative telomere length assay

Telomere length (TL) values were measured from buccal DNA using a validated qPCR assay that determines the relative ratio of telomere repeat (T) copy number to single-copy gene (S) number (T/S ratio) in experimental samples as compared with a reference DNA sample (Cawthon, 2002).

Telomere primers amplify the telomere sequences. The PCR signal is a measure of TL, because the number of telomere primers that can bind the telomeric DNA at the beginning of the PCR is directly proportional to the total summed length of all the telomeres in the cell. T/S = 1 when the unknown DNA is identical to the reference DNA in its ratio of telomere repeat copy number to single copy gene copy number.

5.3.3 Statistics

I tested the hypothesis that preterm birth would be associated with shorter TL using a multivariate linear regression analysis. The outcome variables were TL at birth, term age and 1 year. Variables that could confound the association or be in the causal pathway were added into the model in a hierarchical manner. These variables included characteristics that have been suggested to be associated with TL in previous studies: gender (Benetos et al., 2014) (Entringer et al., 2014), socioeconomic deprivation (Drury et al., 2011), smoking (Valdes et al., 2005) and paternal age (Broer et al., 2013) (Njajou et al., 2007). Also included were variables that were grossly unequal between the groups: breast milk at 3 months and mode of delivery. Male gender was over-represented in the preterm group and TL for this group may be shorter due to male gender being associated with shorter telomeres (Benetos et al., 2014). The unstandardised regression (β) coefficients from these models indicate the number of units that the T/S ratio will change as a result of one-

unit change in the other predictors. Paired samples student's t test was used to compare the mean TL between term and preterm infants where appropriate. Mean TL values were normally distributed. Data are expressed as mean ± SEM. Statistical significance was set at $p < 0.05$ (2 tailed).

5.3.4 Covariates

Socioeconomic deprivation was coded as DEPCAT (deprivation category) score. DEPCAT scores are based on the mother's postcode at booking and obtained from "Carstairs scores for Scottish postcode sectors from the 2001 Census" (McLoone, 2004). The scores are from 1 to 7 where 7 indicates the worst social deprivation. *Maternal smoking* was categorised as current smoker, never smoked, former (stopped pre-pregnancy) or former (stopped during pregnancy). *Labour* was whether the mother experienced labour prior to delivery or not. *Breast milk at 3 months* was whether the infant was receiving any breast milk at 3 months corrected age or not. *Paternal age* was calculated for the time of the infant's birth.

5.4 Results

5.4.1 Relative telomere length at birth and term corrected age

The mean TL at birth was 1.24 ± 0.1 for the term infants and 1.52 ± 0.1 for the preterm infants. The difference in mean TL between preterm infants and term infants at birth, 0.31 95% CI [0.1, 0.6], was significant, $p = 0.02$ (Figure 5.1).

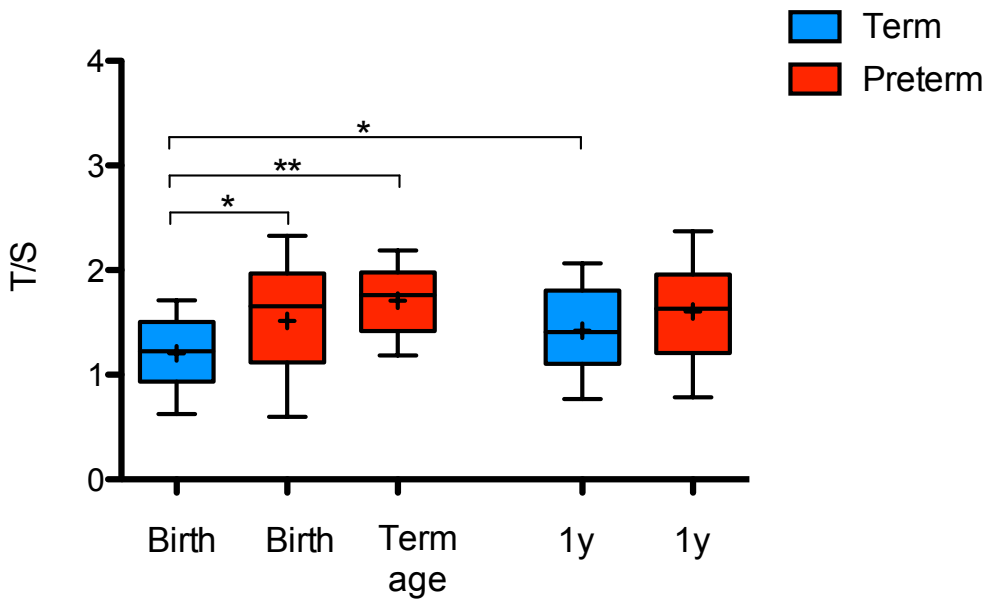


Figure 5.1 Relative telomere length in term and preterm infants over 1 year

Box and whisker plot: 10th to the 90th percentile with the line at median (+ indicates the mean). Term infants at birth ($n = 34$), preterm infants at birth ($n = 31$), preterm infants at term age ($n = 40$), term infants at 1 year ($n = 32$) and preterm infants at 1 year ($n = 36$). *$p < 0.05$ **$p < 0.001$

There was a slight increase in the coefficient for preterm birth with adjustment for gender, social deprivation score and paternal age, β = 0.3, 95% CI [0.03, 0.6], p = 0.03. However, with the addition of maternal smoking and thereafter mode of delivery to the hierarchical model, the difference was no longer significant, β = 0.3, 95% CI [-0.04, 0.5], p = 0.09. None of the covariates (gender, social deprivation, paternal age, maternal smoking or labour) were significant contributors individually. The regression coefficients for all covariates are in Table 5.1.

		β [95% CI]	*t*	*p*
Model 1	Prematurity	0.3 [0.01, 0.5]	2.1	0.04
Model 2	Prematurity	0.3 [0.03, 0.6]	2.2	0.03
	Male	-0.02 [-0.3, 0.3]	-0.2	0.88
	Social deprivation	-0.1 [-0.2, 0.03]	-1.4	0.17
	Paternal age	-0.01 [-0.03, 0.02]	-0.4	0.66
Model 3	Prematurity	0.3 [-0.04, 0.5]	1.7	0.09
	Male	-0.04 [-0.3, 0.2]	-0.3	0.77
	Social deprivation	-0.07 [-1.7, 0.03]	-1.5	0.15
	Paternal age	-0.003 [-0.02, 0.02]	-0.3	0.77
	Maternal smoking	0.2 [-0.01, 0.3]	1.8	0.07
	Labour	-0.2 [-0.4, 0.1]	-1.4	0.18

R^2 = 0.1 for model 2 (p = 0.7) and R^2 = 0.2 for model 3 (p = 0.18)

Table 5.1 Linear model of predictors of TL in preterm infants and term infants at birth

There was also a significant difference between mean TL of preterm infants at term age and term infants at birth, mean difference = 0.5, 95% CI [0.3, 0.7], $p < 0.001$. This remained significant with adjusted analysis, β = 0.6, 95% CI [0.4, 0.8], $p < 0.001$. None of the covariates were significant contributors individually. The regression coefficients for all covariates are in Table 5.2. There was no difference in mean TL between preterm infants at birth and at term corrected age, 0.2, 95% CI [-0.1, 0.5] $t = 1.6$, $p = 0.12$.

		β [95% CI]	*t*	*p*
Model 1	Prematurity	0.5 [0.3, 0.7]	5.2	< 0.001
Model 2	Prematurity	0.6 [0.4, 0.8]	5.0	<0.001
	Male	-0.1 [-0.3, 0.1]	-0.8	0.4
	Social deprivation	-0.02 [-0.1, 0.1]	-0.5	0.61
	Paternal age	0.01 [-0.01, 0.03]	0.9	0.36
	Maternal smoking	-0.02 [-0.1, 0.01]	-0.4	0.7
	Labour	0.1 [-0.1, 0.3]	0.9	0.37

$R^2 = 0.33$ for model 2 ($p = 0.37$)

Table 5.2 Linear model of predictors of TL in preterm infants at term age and term infants at birth

5.4.2 Relative telomere length at 1 year

The mean TL at 1 year was 1.4 ± 0.1 for the term infants and 1.6 ± 0.1 for the preterm infants. This difference was not significant, mean difference = 0.2, 95% CI [0.1, 0.4], $p = 0.16$. In a hierarchical regression model, the difference was significant controlling for gender, maternal smoking and social deprivation score, β = 0.3, 95% CI [0.1, 0.6], $p = 0.02$. However, the significance was lost with the addition of breast milk exposure at 3 months, β = 0.3, 95% CI [-0.03, 0.6], $p = 0.07$. Although social deprivation score did not predict TL at birth, this was included in the model with the view that this may affect TL during infancy. Maternal smoking was included as there was a trend towards this being a significant predictor at birth. Paternal age and mode of delivery were not included at 1 year. Gender was a significant predictor at 1 year

of age with TL being shorter in males: β = -0.3 95% CI [-0.6, -0.02], p = 0.03. None of the other covariates (social deprivation, maternal smoking or breast milk at 3 months) were significant contributors individually. The regression coefficients for all covariates are in Table 5.3.

		β [95% CI]	t	p
Model 1	Prematurity	0.2 [-0.1, 0.4]	1.4	0.16
Model 2	Prematurity	0.3 [0.1, 0.6]	2.3	0.02
	Male	-0.3 [-0.6, -0.02]	-2.2	0.03
	Maternal smoking	-0.1 [-0.2, 0.1]	-1.1	0.28
	Social deprivation	-0.03 [-0.1, 0.1]	-0.7	0.52
Model 3	Prematurity	0.3 [-0.03, 0.6]	1.8	0.07
	Male	-0.3 [-0.5, 0.01]	-1.9	0.06
	Maternal smoking	-0.1 [-0.2, 0.1]	-1.1	0.26
	Social deprivation	-0.03 [-0.1, 0.1]	-0.7	0.48
	Breast milk at 3 months	-0.1 [-0.4, 0.2]	-0.5	0.61

R^2 = 0.1 for model 2 (p = 0.13) and R^2 = 0.1 for model 3 (p = 0.61)

Table 5.3 Linear model of predictors of TL in term infants and preterm infants at 1 year

5.4.3 Change in relative telomere length over 1 year

For the preterm infants, the change in mean TL from term age to 1 year was 0.1, 95% CI [-0.1, 0.4] and this was not statistically significant, t = 1.1, p = 0.29. Similarly, the change in mean TL from birth to 1 year was 0.2, 95% CI [-0.2, 0.6,] and this was not statistically significant, t = 1.0, p = 0.34. However, in the term infants, the mean TL was significantly longer at 1 year than at birth: mean difference = 0.3, 95% CI [0.1, 0.5] t = 2.5, p = 0.02.

5.5 Discussion

This study describes telomere dynamics in both preterm and term infants over the first year of age.

As expected, preterm infants had longer TL at birth than term infants at birth. This is similar to the findings of Menon et al where TL was measured by qPCR in cord blood in a slightly larger group of full term and preterm infants at birth (n = 35 and 69 respectively) but without preterm premature rupture of membranes (pPROM). Where there was pPROM, TL was equivalent to that of the term infants, and the authors speculate that this may be due to oxidative senescence from the pathological processes leading to rupture of membranes (Menon et al., 2012). The preterm infants in my study were born following a variety of obstetric circumstances and therefore included both intact membranes and pPROM. Despite this, TL was longer in the preterm group. In a much smaller study (total n = 26), Friedrich et al did not demonstrate that the average TL was longer in preterm infants compared to term infants, but showed an inverse relationship between TL and gestational age in the preterm infants only (Friedrich et al., 2001) as did Menon et al (Menon et al., 2012). Though extrapolated from single measurements from each preterm infant at birth, this suggests that there is a steady shortening of TL during fetal life. However, the findings of Holmes et al, again in a very small sample (total n = 13) showed steady shortening of TL in the preterm infants during hospital stay and not in the serial measurements from fetuses matched for gestational age (Holmes et al., 2009). Based on this, one might expect preterm infants to have shorter or equivalent TL to full term infants by their expected due date, however none of these previous studies measured TL at this time point.

Here lies the main strength of this study - its longitudinal design. I did not observe shortening of TL in the preterm group postnatally i.e. by term corrected age, TL was equivalent to that of the term infants. It may be regarded that preterm infants mature rather than 'age' until term corrected age. TL in preterm infants did not shorten over the first year. Surprisingly, TL appeared to lengthen in term infants from birth to 1 year, so by 1 year there was no significant difference in TL between the groups.

These results do not suggest accelerated cellular ageing as a result of preterm birth, at least up to one year of age.

Other longitudinal studies have reported telomere lengthening in some individuals (Shalev et al., 2012) (Epel et al., 2009) and there are a number of potential explanations for this. Some have argued that TL can "oscillate" when measured over short periods of time (months) but that it can level out over longer periods (years) (Svenson et al., 2011). Telomere lengthening by telomere synthesis may also be due to up-regulation of telomerase activity, and lifestyle, nutrition and stress are factors that been shown to influence telomerase activity in adults (Ornish et al., 2008) which may have played a role in the relatively healthy term group. Thus, in the term infants in my study, the measured telomere lengthening may be a marker of resilience. As a biomarker of disease risk, a static measure of TL may not perform as well as a measure over time showing either attrition or lengthening of TL.

There are limitations of this study of which tissue type is an important consideration. Many of the published studies involved quantifying TL in leucocytes, however obtaining blood for research in children poses an ethical problem. Buccal cells, on the other hand, are easily accessible. Although TL should be measured in single cell populations as much as possible, saliva samples may not yield purely buccal cells and may include dead or dying cells and leucocytes; both of which could lead to variation in TL measurement. Similarly, peripheral blood has varying proportions of leucocyte subsets, and again this may lead to variation in TL measurement. Two previous studies have reported opposite findings regarding the correlation of TL between buccal cells and leucocytes (Thomas et al., 2008) (Gadalla et al., 2010) in adults. In another study, although TL varied within individuals across different tissues, the rate of attrition was similar across tissues (Daniali et al., 2013). Nevertheless, there is good correlation of TL between skin cells and leucocytes in newborns (Okuda et al., 2002). In children, studies reported negative effects of early life stress on buccal cell TL (Shalev et al., 2012) (Drury et al., 2011) (Mitchell et al., 2014) and showed that a more reactive temperament was associated with shorter TL (Kroenke et al., 2011). These studies re-inforce the potential utility of buccal cells for the study of telomere length.

Various methods for measuring TL, specifically in preterm infants, have been reviewed by Turner et al (Turner et al., 2014). The gold standard method of measuring TL is the Southern blot analysis of the terminal restriction fragments (TRF) (Kimura et al., 2010). We chose to quantify relative TL using the qPCR method and there is a strong correlation between this and the TRF method. (Cawthon, 2002) The primers are designed to only detect and amplify intact telomeric DNA sequences (TTAGGG sequences), whereas the TRF method overestimates TL due to human DNA containing a variable amount of TTAGGG-like repeat sequences (O'Callaghan & Fenech, 2011). Measuring the telomeric repeat region by qPCR can be prone to the interference of primer-dimers and overestimation of TL. Great care and repeated optimisation was undertaken in this study to avoid this. A modification of the qPCR method with the use of an oligomer standard has enabled absolute TL to be calculated as opposed to relative TL (O'Callaghan & Fenech, 2011). Cawthon, who developed the original qPCR method, referred to as a singleplex assay, also described a multiplex qPCR method which is reported to remove the variation in T/S introduced by varying amounts of DNA pipetted and to reduce cost and throughput by halving the number of reactions needed (Cawthon, 2009). It would be desirable to repeat the measurement of TL using these methods.

In summary, preterm infants had longer telomeres at birth compared to term infants at birth, but did not show telomere attrition suggestive of accelerated senescence. In contrast, telomeres lengthened in the term born infants and this may be via a different mechanism. It would be invaluable to measure TL and additionally telomerase levels, at a later time point in this cohort. Telomere attrition is reported to be the fastest in the first 4 years of age (Frenck et al., 1998) (Zeichner et al., 1999), hence a time point thereafter may reveal larger discrepancies between the groups if preterm birth alters the trajectory of telomere attrition in the long term.

The studies so far have focussed on changes in DNA in preterm infants after birth, yet the study of the placenta can yield clues exclusively about processes during intra-uterine life. Interruption of placental support defines preterm birth and may be the outcome of pathological processes in the placenta. Altered DNA methylation may be

implicated with consequences for developmental programming. It also provides an opportunity to study 5-hydroxymethylcytosine in relation to fetal programming, which was recently re-discovered. In the next chapter, I present data on DNA methylation in the placenta and its relationship to size at birth.

Chapter 6: The placenta and developmental programming

6.1 Introduction

The DNA modification, 5-hydroxymethylcytosine (5hmC) has been the subject of much research in recent years. It is a stable modification in its own right and is thought to be an intermediate step in DNA de-methylation (He et al., 2011). However the positive relationship between 5hmC at gene bodies and transcription suggests that 5hmC may be a useful signature of transcriptional state (Mellén et al., 2012). Unlike in blood or buccal DNA, 5hmC is present in the mature placenta (Nestor et al., 2012), however 5hmC in the placenta has not been studied in the context of fetal programming nor as a potential regulator of placental function. The placenta, being the key conduit of nutrients, hormones and oxygen to the fetus (Reik et al., 2003), is a valuable source of information about intra-uterine life. As it is a temporary organ, it is highly accessible to study in sufficient quantity and therefore this tissue has been used to examine the relationship between gene expression and/or 5mC and birth weight (St-Pierre et al., 2012) (Banister et al., 2011) (Guo et al., 2008). Studies using traditional bisulfite conversion methodology would have not been able to technically discriminate 5mC from 5hmC (Huang et al., 2010) and therefore the role of the latter and less well-studied modification may have been underestimated. Moreover, the true abundance of 5mC may not have been accurately captured.

Being rich in imprinted genes, the placenta is the site that serves the parental 'conflict' theory, which suggests that imprinting evolved as a result of competition between the maternal and paternal genomes over provision of maternal nutrients to the fetus (Moore & Haig, 1991). Imprinted genes are epigenetically regulated, in particular by DNA methylation and are therefore popular candidates for study in the context of early life programming.

Most prior studies have compared IUGR/SGA vs. AGA pregnancies, however we know that morbidity and mortality is graded across the normal birth weight range with the lightest and heaviest babies being most affected (Basso et al., 2006).

Gestational age also affects gene expression and DNA methylation and may have confounded previous results (Novakovic et al., 2011) (Kumar et al., 2012). I therefore set out to study gene expression, 5mC and 5hmC in term placenta in relation to fetal growth across the normal range of birth weight.

6.2 Hypothesis

I aimed to test the hypothesis that expression in the placenta of candidate imprinted genes that mediate fetal growth associates with size at birth. In addition, since a number of non-imprinted genes that are expressed in the placenta have also been implicated in developmental programming, I also wished to study the expression of candidate non-imprinted genes that mediate fetal growth in the placenta in relation to size at birth.

Thereafter, I aimed to study epigenetic control mechanisms that might affect gene dosage using new affinity based techniques that would distinguish between 5mC and 5hmC allowing me to study their individual relationship with birth weight.

6.3 Methods

6.3.1 Subjects and tissue collection

Placental samples from 72 women with a singleton pregnancy at full term (>37 weeks) representing a wide variation in birth weight (range 2140 – 4750g) were obtained from the Edinburgh Reproductive Tissue Bio-bank (ERTBB). Women gave written informed consent and trained staff carried out sampling according to a standard operating procedure. The samples were collected straight after delivery from the centre of the placenta away from the cord and large blood vessels but from the fetal side. Samples were snap frozen or processed in RNA*later* Stabilising Reagent (Qiagen, Crawley, UK), as appropriate at the point of collection. Pregnancies complicated by congenital abnormalities or diabetes (pre-pregnancy or gestational) were excluded as these might alter fetal growth. Deliveries by Caesarean section predominate samples collected for the ERTBB due to ease of obtaining informed consent.

Samples were chosen by manually reviewing the demographic records held by the ERTBB. Samples were chosen with the aim to form equal groups comprising infants who were small for gestational age (SGA), appropriate for gestational age (AGA) and large for gestational age (LGA). SGA is defined as birth weight less than 10th percentile for gender and age. AGA is defined as birth weight greater than the 10th percentile but less than the 90th percentile and LGA is defined as greater than the 90th percentile for gender and age. Baseline characteristics are listed in Table 6.1 and divided into SGA, AGA and LGA for the purpose of describing the samples.

	SGA (*n* = 21)	AGA (*n* = 24)	LGA (*n* = 27)	All (*n* = 72)
Birth weight, g, mean (SD)	2515 (201)	3250 (383)	4401 (189)	3467 (829)
Birth weight SD score, mean (SD)	-1.85 (0.4)	-0.36 (0.8)	1.97 (0.4)	0.08 (1.7)
Birth weight centile, mean (SD)	4.4 (3.1)	38.3 (26.4)	96.9 (2.4)	50.38 (41.6)
Gestation, weeks, mean (SD)	38.8 (1.1)	39.2 (1.0)	39.9 (1.0)	39.4 (1.1)
Male, *n* (%)	7 (33.3)	10 (41.7)	15 (55.6)	32 (44.4)
Maternal age, years, mean (SD)	31.8 (4.6)	34.8 (5.5)	34.2 (4.4)	33.7 (4.9)
Caucasian ethnicity, n (%)	16 (76.2)	23 (95.8)	25 (92.6)	64 (88.9)
Primiparous, *n* (%)	7 (33.3)	8 (33.3)	3 (11.1)	18 (25.4)
BMI, kg/m^2, mean (SD)	28.7 (8.2)	23.2 (2.9)	29.9 (6.1)	27.3 (6.6)
Pre-eclampsia, *n* (%)	4 (19.1)	0	0	4 (5.6)
Hypertension during pregnancy, *n* (%)	1 (4.8)	0	1 (3.7)	2 (2.8)
Smoking during pregnancy, *n* (%)	4 (19.1)	1 (4.2)	0	5 (6.9)
Caesarean section, *n* (%)	16 (76.2)	22 (91.7)	27 (100)	66 (91.7)

Table 6.1 Baseline characteristics

6.3.2 Laboratory methods

Total RNA was extracted using the Qiagen RNeasy Fibrous Tissue Minikit (Qiagen, Crawley, UK), following the manufacturer's instructions. High quality RNA as assessed by the 2100 Bioanalyser system (Agilent Technologies, Cheshire, UK) was available for 64 samples.

The Lightcycler 480 system (Roche, Burgess Hill, UK) was used for qPCR. A combination of inventoried assays (*Taq*man Gene Expression Assays, Applied Biosystems (ABI), Life Technologies, Paisley, UK), and custom made assays Universal Probe Library assays (UPL) (Roche, Burgess Hill, UK) and Invitrogen (Life Technologies, Paisley, UK) with SYBR Green I technology were used. Details and accession numbers are in Table 2.8.

Of the four candidate reference genes, *YWHAZ*, *TBP, GAPDH* and *SDHA, YWHAZ* was chosen as the most stable using Normfinder (Andersen et al., 2004) and geNorm^{PLUS} as part of the qbase^{PLUS} software (Eclipse v 3.7, Biogazelle, Zwijnaarde, Belgium). Gene expression of the imprinted genes *IGF2, H19, CDKN1C, GRB10, PHLDA2, DLK1, PEG10* and the non-imprinted genes, *GR, HSD11β2, IGF2R* and *PPARγ* was quantified using qPCR.

High quality RNA-free DNA was obtained available for 72 samples. DNA was sonicated to the appropriately sized fragments. Enrichment of 5mC was achieved using the Active Motif MethylCollector Ultra kit. Enrichment of 5hmC was achieved using a hydroxymethyl DNA immunoprecipitation technique. Percentage enrichment at the DMRs controlling the expression of *IGF2* and *H19*: *IGF2 DMR0, IGF2 DMR2, H19 ICR, H19* gene body, *H19* promoter and the DMR controlling the expression of *CDKN1C* and *PHLDA2*: *KvDMR* was analysed using qPCR.

6.3.3 Statistics

Weight measurements were adjusted for gender by converting to standard deviation scores (z-scores) and centiles using LMSgrowth, a Microsoft Excel Add-in to access growth references that define the UK-WHO growth charts.

Values for relative gene expression and percentage enrichment were log transformed using natural logs to achieve a normal distribution.

Multivariate linear regression analysis was used to test the hypothesis that birth weight is associated with altered gene expression, 5mC and 5hmC. Covariates that could confound the association or be in the causal pathway were added into the model. The outcome variable was birth weight SD score. The standardised regression (β) coefficients from these models indicate the number of standard deviations in relative gene expression / percentage enrichment will change as a result of one standard deviation change in the other predictors. Statistical significance was set at $p < 0.05$ (2 sided).

6.3.4 Covariates

Birth weight is a complex outcome, which is influenced by many processes: gestational age, maternal weight, parity, illness, ethnicity and infant sex. Maternal *smoking* was whether the mother smoked during pregnancy. *Maternal BMI* was included as it has been shown in our department that it is associated with changes in gene expression. Other covariates included were *pre-eclampsia* and *parity*. Almost all the women were Caucasian. Ethnicity can influence fetal growth, but we chose not to include this as a covariate. The non-Caucasian women were a mix of Asian, Arabic and Chinese origin and adjustment for ethnicity would require assumptions as to the direction of fetal growth. Furthermore, ethnicity was self-reported, thus unreliable, and does not necessarily reflect the ethnicity of the fetus. Paternal ethnicity was not held on file by the Bio-bank.

6.4 Results

6.4.1 Gene expression

		r	*p*	Standardised β	Adjusted *p*
Paternally imprinted	*IGF2*	0.3	0.02	0.2	0.10
	DLK1	0.1	0.64	0.03	0.84
	PEG10	-0.2	0.21	-0.1	0.35
	ZIM2	0.2	0.23	0.1	0.33
Maternally imprinted	*H19*	0.2	0.08	0.2	0.14
	CDKN1C	-0.3	0.01	-0.3	0.04
	PHLDA2	0.2	0.16	0.2	0.16
	GRB10	0	0.97	0.1	0.39
Non-imprinted	*HSD11β2*	0.2	0.17	0.2	0.20
	GR	0.1	0.36	0	0.98
	IGF2R	0.04	0.78	0.03	0.80
	PPARγ	0.2	0.15	0.2	0.21

Table 6.2 Relationship between birth weight SD score and relative expression of imprinted and non-imprinted genes

Pearson correlation coefficient, *r*, for unadjusted relationship and standardised β values adjusting for maternal BMI, parity, pre-eclampsia and maternal smoking.

There was a positive correlation between birth weight SD score and relative expression of *IGF2*, $r = 0.3$, $p = 0.02$, which was significant when adjusted for maternal BMI, parity and pre-eclapmsia, standardised $\beta = 0.3$, $p = 0.04$, but not when smoking was added into the model, standardised $\beta = 0.2$, $p = 0.1$ (Tables 6.2 and 6.3). Mean relative expression of *IGF2* was 0.3 ± 0.1 compared with non-smokers, 1.7 ± 0.4. The mean difference, 1.4, 95%CI [0.6, 2.2], was significant $t = 3.6$, $p = 0.001$. However, the number of women who smoked was small ($n = 4$).

	Predictors	β [95% CI]	Standardised β	Adjusted *p*
Model 1	*IGF2* mRNA	0.4 [0.1, 0.8]	0.3	0.02
Model 2	*IGF2* mRNA	0.4 [0.02, 0.7]	0.3	0.04
	Maternal BMI	0.02 [-0.04, 0.1]	0.1	0.56
	Parity	0.02 [-0.5, 0.5]	0.01	0.94
	Pre-eclampsia	-2.1 [-4.3, 0.2]	-0.2	0.07
Model 3	*IGF2* mRNA	0.3 [-0.1, 0.6]	0.2	0.10
	Maternal BMI	0.01 [-0.1, 0.1]	0.1	0.67
	Parity	0.2 [-0.3, 0.7]	0.1	0.41
	Pre-eclampsia	-2.2 [-4.3, -0.1]	-0.2	0.04
	Maternal smoking	-2.3 [-3.9, -0.7]	-0.4	0.01

R^2 = 0.15 for model 2 (p = 0.266) and R^2 = 0.26 for model 3 (p = 0.006)

Table 6.3 Linear models of predictors of birth weight SD score

There was a negative correlation between birth weight SD score and the relative expression of *CDKN1C*, r = -0.3, p = 0.01. The relationship remained significant after adjustment for maternal smoking, pre-eclampsia, BMI and parity, standardised β = -0.3, p = 0.04. None of the other genes tested varied significantly with birth weight SD score. Table 6.2

6.4.2 5-methylcytosine (5mC)

Percentage enrichment of 5mC at *IGF2 DMR0* was independently associated with birth weight SD score in adjusted analyses, standardised β = 0.3, p = 0.02. 5mC at the *H19* promoter also showed a positive relationship with birth weight SD score, r = 0.3, p = 0.048, however it was not significant after adjustment for confounders, standardised β = 0.2, p = 0.06. There was a strong relationship between birth weight and 5mC at the *KvDMR*, standardised β = 0.3, p = 0.02. Percentage enrichment of 5mC at *IGF2 DMR2* and 3 regions of *H19 ICR* studied (*H19 DMR*, *CTCF3* and *CTCF6*) was not significantly related to size at birth. Table 6.4

	r	*p*	Standardised β	Adjusted *p*
IGF2 DMR0	0.4	0.002	0.3	0.02
IGF2 DMR2	0.1	0.39	0.1	0.62
H19 DMR	0.1	0.39	0.1	0.46
H19 CTCF3	-0.1	0.53	-0.1	0.49
H19 CTCF6	0.2	0.20	0.1	0.50
H19 Promoter	0.3	0.05	0.2	0.06
KvDMR	0.3	0.02	0.3	0.02

Table 6.4 Relationship between 5mC and birth weight SD score

I also analysed the correlations between DNA methylation and gene expression. There was a trend towards a positive correlation between 5mC at *IGF2 DMR0* and relative expression of *IGF2*, $r = 0.3$, $p = 0.06$. There were no significant relationships between gene expressions and 5mC at the other regions studied. Table 6.5.

		r	*p*
IGF2 mRNA	*IGF2 DMR0*	0.3	0.06
	IGF2 DMR2	0.2	0.20
	H19 DMR	0.2	0.14
	H19 CTCF3	0.1	0.59
	H19 CTCF6	0.1	0.67
H19 mRNA	*H19* promoter	-0.2	0.12
CDKN1C mRNA	*KvDMR*	0.04	0.79

Table 6.5 Relationship between gene expression and 5mC

6.4.3 5-hydroxymethylcytosine (5hmC)

Percentage enrichment of 5hmC within the *H19* gene body correlated with birth weight SD score in adjusted analyses, standardised β = 0.2, $p = 0.04$. Table 6.6.

	r	*p*	Standardised β	Adjusted *p*
H19 gene body	0.3	0.02	0.2	0.04
IGF2 DMR0	0.2	0.17	0.2	0.12
IGF2 DMR2	0.01	0.95	-0.1	0.58
H19 CTCF6	0.1	0.48	0.03	0.81
H19 promoter	-0.1	0.59	-0.1	0.55
KvDMR	0.03	0.80	0.01	0.94

Table 6.6 Relationship between 5hmC and birth weight SD score

Percentage enrichment of 5hmC at the *H19* gene body was taken as the average of 3 individual regions.

	r	*p*	Standardised β	Adjusted *p*
H19 genic 1	0.3	0.004	0.3	0.002
H19 genic 3	0.1	0.25	0.1	0.47
H19 genic 4	0.2	0.05	0.2	0.20

Table 6.7 Relationship between 5hmC at the individual *H19* genic regions and birth weight SD score

There was, however, no relationship between relative expression of *H19* mRNA and percentage 5hmC enrichment at any of the *H19* genic regions or the average of these or at the *H19* promoter. Table 6.8

	5hmC	*r*	*p*
H19 mRNA	*H19* genic average	-0.1	0.44
	H19 genic 1	-0.03	0.83
	H19 genic 3	-0.2	0.15
	H19 genic 4	-0.1	0.64
	H19 promoter	-0.2	0.17

Table 6.8 Relationship between relative expression of *H19* and 5hmC at *H19* gene body and *H19* promoter

Despite the lack of significant association between birth weight SD score and 5hmC at any other region studied, Table 6.6, it was notable that 5hmC was enriched at all of those regions, Figure 6.2, compared with enrichment of 5mC, Figure 6.1. 5hmC is relatively depleted at *GAPDH* and this was used as the negative control. 5mC is also depleted at *GAPDH* with no detectable amplification in the qPCR reaction.

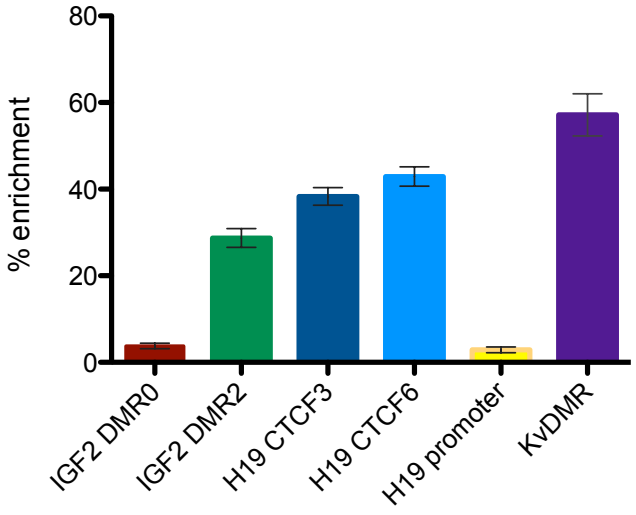


Figure 6.1 Percentage enrichment of 5mC at regions of interest

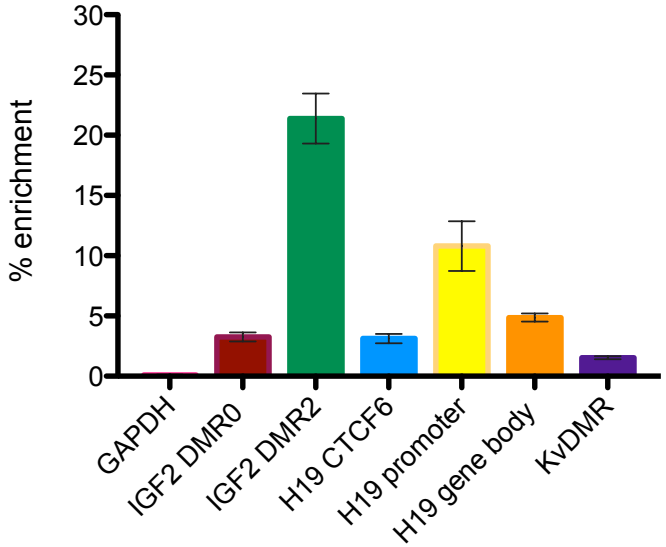


Figure 6.2 Percentage enrichment of 5hmC at regions of interest

6.5 Discussion

My study demonstrated relationships between the expression of two imprinted genes at the same cluster (one maternally and the other paternally imprinted) at 11p15.5 in the placenta and birth weight and thereafter variation in 5mC and 5hmC at known regulatory loci. Few prior studies have examined both gene expression and 5mC and none have studied 5hmC with respect to developmental programming.

We observed variation in expression at imprinted genes as opposed to the non-imprinted genes with birth weight. This is probably not unexpected. Genomic imprinting is fundamentally controlled by DNA methylation (Li et al., 1993) and thus is potentially vulnerable to environmental factors during development such as nutrition. On the other hand, as proposed by Moore and Haig, imprinting evolved to balance nutrient provision to the fetus, i.e. how much an offspring receives/extracts from its mother at the expense of its siblings (Moore & Haig, 1991). They predicted, correctly, that imprinted genes would affect placental growth and fetal growth, but also have further-reaching influences on neonatal behaviour, metabolism and postnatal growth (Fowden et al., 2011) (Tycko & Morison, 2002). Not only is the placenta abundant with imprinted genes, but genes have been identified that are solely imprinted in the placenta (Court et al., 2014) (Coan et al., 2005), one notable example was DNA methyltransferase 1 (*DNMT1)* (Das et al., 2013). Interestingly, patterns of imprinting in placental tissue adopts a different profile to somatic tissues (Court et al., 2014). So, imprinted genes in the placenta could be poised to adapt placental function to changing environmental conditions and can offer mechanisms of disease programming.

The positive relationship between the relative expression of *IGF2* and birth weight did not persist after adjusting for maternal smoking. Although the number of women who smoked was small, our data suggests that maternal smoking may explain part of the relationship. This is in line with recent data showing that maternal smoking associates with reduced DNA methylation and expression of *IGF2* in human fetal liver, with altered availability of methyl donors as a probable cause (Drake et al., 2015). Data from mouse models in which the lack of placental IGF2 leads to fetal

growth restriction and smaller placentae confirm that placental *IGF2* is a potent growth factor (DeChiara et al., 1991) (Constância et al., 2002). However, in humans, similar studies to ours have yielded conflicting results. Three studies comparing gene expression between SGA/IUGR vs. AGA babies showed higher *IGF2* expression in the latter group (McMinn et al., 2006) (Guo et al., 2008) (Kappil et al., 2015) and similarly in a fourth, however in chorionic villus samples (Moore et al., 2015). Some studies showed no relationship (Apostolidou et al., 2007) (Lim et al., 2012) (Turan et al., 2010) whilst one demonstrated raised levels of mRNA in SGA/IUGR vs AGA (Börzsönyi et al., 2011). In contrast to similar studies (McMinn et al., 2006) (Lim et al., 2012) (Apostolidou et al., 2007) (Diplas et al., 2009), we found no association between relative expression of *PHLDA2* and birth weight. The conflicting results may be due different methodology (AGA vs. SGA or IUGR rather than across the birth weight range, candidate gene vs. genome wide approach, varying sample sizes or variation in sampling from the placenta) and several studies did not control for gestation and gender. None of the studies reporting gene expression detailed any objective assessment of reference genes.

The increased enrichment of 5mC at *IGF2 DMR0* with increased birth weight may point towards a role of IGF2 in controlling fetal growth (Murrell et al., 2008). *IGF2 DMR0* is normally methylated on the paternal allele and is reported to show reciprocal patterns of methylation in some cases of Beckwith Wiedemann Syndrome (BWS) and Silver Russell Syndrome (SRS). In BWS, which is associated with increased fetal growth, *IGF2 DMR0* is hypermethylated on the paternal allele in *cis* with hypermethylation at the *H19 ICR*. The opposite is the case in SRS (Murrell et al., 2008), which is associated with IUGR. Additionally, we saw a trend towards a correlation between 5mC at *IGF2 DMR0* and relative expression of *IGF2*. It is important to note that *IGF2 DMR0* is reported to house the promoter for the *IGF2* P0 transcript (Monk et al., 2006). Our qPCR assay for *IGF2* would have measured all known transcripts, including the P0 transcript. In mice, the P0 transcript is expressed exclusively in the labyrinthine trophoblast layer (Constância et al., 2002) where it regulates the placenta's capacity for diffusional exchange and P0-null mice have IUGR (Sibley et al., 2004). In humans, P0 is differentially expressed in term

placenta, but also in fetal skeletal muscle and widely in adult tissues (Monk et al., 2006). Therefore, it is possible that 5mC at *DMR0* may alter P0 transcription and potentially influence metabolism in the neonate and in later in life.

There was a strong inverse relationship between the relative expression of *CDKN1C* and birth weight. This association has been observed in one prior study of the placenta comparing IUGR vs AGA (McMinn et al., 2006). *CDKN1C*, also known as $p57^{KIP2}$, is maternally expressed (Matsuoka et al., 1996) and encodes a cyclin dependent kinase inhibitor which induces cell cycle arrest (Lee et al., 1995). In mice, *cdkn1c* knockout or loss of function mutations modelled BWS with enhanced cell differentiation and proliferation (Zhang et al., 1997) (Tunster et al., 2011), but also placental overgrowth (Takahashi et al., 2000). Indeed, *cdkn1c* is expressed in a tissue specific manner with high levels in the placenta (Lee et al., 1995). As placental weight is a key determinant of birth weight (Roland et al., 2012), it is likely that *CDKN1C* in humans also plays a role in regulating fetal growth via its effect on placental development. Whilst mutations at *CDKN1C* occur in a small proportion of BWS (Hatada et al., 1996), in the majority there is loss of methylation at *KvDMR*, an intron on *KCNQ1*, on the maternal allele (Lee et al., 1999) (Smilinich et al., 1999) with associated silencing of *CDKN1C* (Diaz-Meyer et al., 2003). A non-coding RNA (*KCNQ1OT1*), normally expressed only from the paternal allele of *KCNQ1* (Lee et al., 1999) (Smilinich et al., 1999), is thought to cause the bi-allelic silencing of *CDKN1C* in *cis* (Mancini-DiNardo, 2003) (Figure 1.2) The converse findings have been reported in SRS (Schönherr et al., 2007).

In this light, it is surprising to have seen a positive correlation between 5mC at *KvDMR* and birth weight, which is in the opposite direction to that which might be expected. There was also no relationship between the relative expression of *CDKN1C* and 5mC at *KvDMR*. This may indicate that the regulation of *CDKN1C* is more complex. Indeed, data indicates that *KvDMR* has methylation sensitive CTCF binding sites, independent of the *KCNQ1OT1* promoter, with chromatin insulating properties, and that there may also be transcriptional enhancers just upstream of the promoter (Fitzpatrick et al., 2007). The relative expression of this anti-sense transcript has not been measured in this study but may yield useful data.

Where there was loss of methylation at *KvDMR* resulting in BWS, it was found to be independent of loss of methylation at *IGF2* (Lee et al., 1999). However, CDKNIC and IGF2 peptides appear to antagonise actions on cell cycle progression (Caspary et al., 1999) and additionally, *cdkn1c* expression has been shown to be affected by increased IGF2 protein (Grandjean et al., 2000). So, as these two regions are not entirely independent, one may not expect perfectly congruent results in a group of normal but heterogeneous pregnancies, but may show elements of both. Where 5mC and gene expression were compared in the several studies of the human placenta, no relationship between the two was seen (Table 1.3) apart from Koukoura et al (Koukoura et al., 2011). A relationship was observed by Lim et al but this was not in placenta but in the umbilical cord which is of different embryonic origin (Lim et al., 2012). This suggests that there are alternative mechanisms and/or additional layers of epigenetic modifications driving changes in gene expression. Histone modifications influence chromatin state and accessibility to transcription factors and other gene regulatory proteins. DNA methylation can, in turn, influence histone modifications closely associated with the locus, for example stimulation of histone deacetylation by methyl-CpG binding proteins. Thus transcriptional state depends on a certain balance between the interplay of factors that activate or silence transcription. Feedback loops also exist between several chromatin modifications that are either mutually re-inforcing or mutually inhibitory and direct transcriptional state (Jaenisch & Bird, 2003). Furthermore, histone modifying enzymes utilise co-factors or substrates belonging to pathways of intermediary metabolism and research suggests that histone modifications (and thereafter, gene expression) can alter dynamically in response to the metabolic status of the cell (Gut & Verdin, 2013). Another modification could be partially methylated domains, which have been recently identified in normal placental tissue and known to associate with gene repression and inactive chromatin marks (Schroeder et al., 2013).

5hmC has been reported to be enriched in the bodies of highly expressed genes (Song et al., 2011). I was, however, not able to demonstrate a relationship between 5hmC at any of the chosen sites and relative expression of the corresponding mRNA. However, the positive relationship between increasing enrichment of 5hmC in the

H19 gene body and birth weight that I describe suggests more complex processes are implicated in modulating fetal growth. The function of 5hmC appears to be dependent on cell type: distinct roles for 5hmC in neurons and embryonic stem (ES) cells have been proposed (Mellén et al., 2012). It is possible the role of 5hmC in the placenta is very different from these described. As the enrichment of 5hmC in the placenta is several fold less than in the brain or ES cells (Nestor et al., 2012), it is also possible that in some genomic regions and some tissue types, 5hmC is indeed a passive intermediate in the demethylation pathway. That we could not demonstrate a significant relationship between 5hmC at any of the regions studied with gene expression may reflect this. Unlike 5mC, whether 5hmC is maintained during mitosis with high fidelity is unknown (Shen & Zhang, 2013) - it may be a short-lived entity. Thus the pattern of enrichment we describe may be of a snap shot in time (at full term when the placenta is nearing the end of its life) rather than a legacy of earlier intra-uterine events affecting fetal growth.

In affinity based analyses of human and mouse ES cell and neuronal genomes, in addition to gene bodies of actively transcribed genes, 5hmC maps at or around promoters, transcription start sites, CpG islands as well as inter-genic regions (*cis*-regulatory elements such as enhancers and insulator binding sites) (Shen & Zhang, 2013). Of the regions we studied, the greatest enrichment was at *IGF2 DMR2* (an intra-genic region), the second highest was at the *H19* promoter and thirdly the *H19* gene body. In addition to the *H19* gene body, the presence of 5hmC at the *H19* promoter, and sites corresponding to *H19 ICR* (insulator-binding site) and *IGF2 DMR2* have been demonstrated by others (Nestor et al., 2012). We have confirmed this and shown 5hmC enrichment at further sites: *IGF2 DMR0* (housing the *IGF2* promoter) and *KvDMR* (an intra-genic site). Enrichment at all of these sites prohibits us from using a more quantitative technique, such as pyrosequencing, for further study because it would not distinguish between 5mC and 5hmC (Huang et al., 2010). The results of DNA methylation studies, past and present, of these and other popular targets in placental tissue need to be interpreted with this in mind. The MBD protein affinity assay we used is specific for 5mC - it does not detect 5hmC (Jin et al., 2010). Although we can be confident about the interpretation of our data, further studies

could utilize newer techniques including oxidative bisulphite sequencing to study both 5mC and 5hmC at single nucleotide resolution (Booth et al., 2012). Utilising the same technique for both modifications may allow better determination of the relative amount of 5mC to 5hmC to each other at each region.

The immunoprecipitation technique to capture 5hmC has a higher noise to signal ratio than the newer chemical capture technique and thus is less specific for 5hmC (Thomson et al., 2013). Exemplified here is the minimal apparent detection of 5hmC, but not 5mC, at *GAPDH* where the former is far less abundant. It also required DNA fragments that were relatively large (approximately 1 kilobase) and thus it is not possible to determine the modification status of individual CpGs (Thomson et al., 2013). Techniques such as oxidative bisulphite sequencing or TET assisted bisulphite sequencing (Yu et al., 2012) offer single nucleotide resolution. Both do not require an enrichment step before sequencing, so the final result reflects the absolute 5hmC at each site (Shen & Zhang, 2013). They may, in the fullness of time, become cost-effective and further work could take advantage of this. Nevertheless, the immunoprecipitation technique is an acceptable method and has been shown to display highly reproducible patterns of 5hmC enrichment between individuals (Thomson et al., 2013).

Other limitations in this study need to be considered. The placental samples from the SGA babies comprise those that are constitutionally small or growth restricted due to a variety of pathologies such as smoking or pre-eclampsia. We aimed to correct for this in the regression model. Limiting the study to full term pregnancies meant that many cases of growth restriction were excluded as these are often delivered before 37 completed weeks. Some cases of fetal growth restriction do not result in SGA and may be classified as AGA. Choosing to analyse variation in 5mC, 5hmC and gene expression across the birth weight range as opposed to SGA vs AGA circumvents this problem, but does not specifically identify patterns associated with fetal growth restriction. Samples were not randomly chosen and I was not blinded to the size category of each sample when performing laboratory assays or analysing data, but groups were mixed to avoid batch effect.

Analysis of placental tissue may be confounded by contamination with maternal tissue and heterogenous cell lines. Placental sampling was en block and tissue was not washed prior to freezing, thus may have been contaminated with maternal blood. For the Bio-bank, sampling of chorionic villi is from the fetal surface to avoid contamination with maternal decidua, as recommended by Hogg et al (Hogg et al., 2014). A preferable option would be to select chorionic villous trophoblast cells by immunoselection as these cells form the feto-maternal barrier, as opposed to the extra-villous component of which includes interstitial and endovascular trophoblast. DNA methylation levels are known to vary between the cellular components and correcting for multiple cell types is not currently feasible (Hogg et al., 2014). Structural abnormalities occur in placentas with fetal growth restriction as underlying pathological processes such as pre-eclampsia are fundamentally abnormalities of trophoblast invasion. Abnormalities include villus regression, volume decrease in the fetal and maternal blood spaces and reduction in the surface area of fetal capillaries (Burton, 2011). This can alter the cellular composition of samples collected and hence also the methylation and gene expression profiles. The shortcomings associated with examining placental samples as a whole would have been encountered in almost all prior studies to date detailed in Tables 1.1 to 1.4. DNA methylation and gene expression were assayed in this study in 2 different tissue samples and may contribute to the discrepancy we observed. Integrity of tissue placental tissue is also a key determinant. It has been recognised that the placenta degrades quickly and can interfere with RNA quality and ultimately the results of gene expression (Lanoix et al., 2012). Almost all the tissues collected were from Caesarean section deliveries since this is mostly what is available in the Bio Bank but this also means the samples were collected soon after delivery for snap freezing. I also only used RNA that was of high quality for the assays. Further, following the MIQE guidelines (Bustin et al., 2009), I analysed reference genes objectively. This is crucial for gene expression studies involving human tissue. There may be concerns regarding intra-placental variation in gene expression. However, a study suggests that there is more variation between individuals than within an individual (Turan et al., 2010). Nevertheless, placental sampling location was consistent to minimise variation, again as per Hogg et al (Hogg et al., 2014). We have observed many of the

recommendations for reporting DNA methylation data of the human placenta – gestational age and fetal sex were adjusted for using standard deviation scores, maternal characteristics detailed (bar alcohol consumption and medication use), β and percentage enrichment values were reported as well as negative findings (Hogg et al., 2014).

Obtaining samples from a Bio-bank meant being restricted to information already gathered. By the nature of information collected at the time of delivery, it is limited to the details about pregnancy and the newborn at birth. There may be newborns with as yet undiagnosed congenital anomalies that would ordinarily not be included in this study. The effect of social deprivation on gene expression, 5mC and 5hmC could not be measured or adjusted for this as the information needed to assess this is not held by the ERTBB and it is not within the remit of the current ethics approval to obtain this information. There is a high heritability of birth weight i.e. mother's birth weight is a good predictor of offspring birth weight. This information is not available to us, however, we did control for maternal BMI. We did not look at SNPs that may influence the expression of genes at these domains.

In summary, we present further evidence that altered gene expression and DNA methylation (5mC) at imprinted loci could provide a link between the supply of nutrients, fetal growth and ultimately, programming of later disease. We further suggest that 5hmC may also play a role in modulating this process. Whilst there was no relationship with gene expression it must be noted that we are comparing single time point measurements of gene expression at term vs. epigenetic changes that may have occurred much earlier in development. Equally, as 5mC and 5hmC are considered to be dynamically related to each other, the enrichment patterns we describe could also be snap-shots within these processes. Clearly, mechanisms mediating gene expression other than methylation must be considered but imprinted genes remain popular targets for studying molecular mechanisms of programming. Moreover, as DMRs are established during gametogenesis and post-fertilisation, they point towards a non-genetic heritability of birth weight and thereby disease risk.

Chapter 7: Conclusion

Following preterm birth, the neurological sequelae of sensor-motor and cognitive deficits in childhood are well documented (Marlow et al., 2005) (MacKay et al., 2010) and the increased prevalence of psychiatric disorders in later life is becoming more apparent (Nosarti et al., 2012). Also emerging are risk factors for cardio-metabolic disease in young adults following very preterm birth (Parkinson et al., 2013) (Tinnion et al., 2014). The advancements in neonatal intensive care resulting in increased survival following very preterm birth, coupled with the increasing incidence of preterm birth worldwide (Blencowe et al., 2013) means we have yet to encounter the true extent and impact of the associated morbidity in later adulthood.

The spectrum of morbidity and risk factors is similar to that described by Barker and colleagues for full term babies who were low birth weight or growth restricted in utero and where the phenomenon is known as 'early life programming' (Barker, 1998) (Gillman, 2005). The two principal theories are fetal undernutrition (Hales & Barker, 2001) and overexposure to glucocorticoids (Edwards et al., 1993), which are not mutually exclusive. Both of them are relevant in the context of preterm birth; manifesting as failure to achieve the expected in utero growth trajectory after preterm delivery, followed up by catch-up growth, altered adiposity and altered HPA axis activity (Cole et al., 2014) (Hack et al., 2003) (Thomas et al., 2011) (Tegethoff et al., 2009). Evidence is building that these features may operate in the pathogenesis of disease risk in preterm infants as in growth restricted term infants (Dalziel et al., 2005b) (Rotteveel et al., 2008).

In this thesis, by establishing a cohort of very preterm and full term infants and describing those phenotypic characteristics implicated in early life programming, I have explored specific molecular mechanisms (DNA methylation and telomere attrition) that may mediate these processes. I have also examined the role of DNA methylation in the placenta in developmental programming.

7.1 Growth

The growth pattern displayed by the preterm group was similar to that described in the literature: downward deviation in weight standard deviation score from birth to term corrected age (Cole et al., 2014), with relative head sparing (Uthaya et al., 2005) (Cockerill et al., 2006) reminiscent of fetal growth restriction (Kramer et al., 1989). Preterm infants remained, on the whole, smaller than their full term counterparts over the first year (Roze et al., 2012). Whilst they did not demonstrate catch-up growth, as evidenced by greater velocity of weight gain than the term group, length was equivalent in both groups by 1 year. Moreover, catch-up growth has been shown to continue during childhood and adolescence (Hack et al., 2003).

Preterm infants had greater percentage body fat at term equivalent age as measured by air displacement plethysmography (densitometry), which normalised (by acquisition of relatively more lean mass) by 3 months corrected and this pattern has been widely reported using this device (Roggero et al., 2009) (Carberry et al., 2010) (Ramel et al., 2011) (Simon et al., 2013). This probably reflects, at least in part, current nutritional practices during neonatal care and post-discharge (Simon et al., 2014) (Gale et al., 2012). Whether changes in body composition beyond 6 months can be detected by densitometry is unknown as this is not practical using the PEAPOD body composition system, but the Pediatric Option accessory to the adult system (BODPOD) can assess children as small as 12kg, which is typically 2 years of age, and up to 6 years of age (http://www.cosmed.it/en/products/related-products/options/bod-pod-pediatric-option, accessed 1 July 2015). It is likely that data on body composition will appear for ex-preterm children using this device. Aberrant adiposity at term corrected age has been attributed, at least in part, to raised cortisol levels (Uthaya et al., 2005). Likewise, it would be interesting to observe the trajectory of cortisol dynamics in childhood and later years following the low salivary cortisol levels measured at 3 months (possible suppression from antenatal glucocorticoids or from raised endogenous cortisol due to the stressors associated with neonatal intensive care). If an alteration in the 'set point' of the HPA axis is demonstrated, this could predispose to cardio-metabolic risk factors such as central adiposity (Reynolds, 2013b), but also cognitive impairment (Reynolds et al., 2010).

7.2 DNA methylation

It is speculated that epigenetic processes, such as DNA methylation (5-methylcytosine or 5mC), can transfer early environmental signals into changes in metabolic, endocrine, and neural regulatory pathways. These changes, in turn, may be responsible for distinct profiles of growth, metabolism, development and behavior (Jirtle & Skinner, 2007). I chose imprinted genes as candidates as they are known to be involved in these processes and 5mC is fundamental in the control of their expression (Li et al., 1993). Specifically, 5mC at DMRs controlling the expression of *IGF2*/*H19* are normally distributed traits with heterogeneity following early life stress in humans (Heijmans et al., 2007) (Heijmans et al., 2008) (Drake et al., 2012). By term equivalent age, preterm infants had decreased 5mC at both *IGF2 DMR2* and the *H19 ICR* compared with term infants at birth. The changes are in the direction that would predict a reduction in expression of *IGF2* and this could be one explanation for the observed growth faltering. There were no persistent differences in 5mC at 1 year when the growth parameters were not as discrepant. Given the analyses were in buccal cells, reduced *IGF2* expression cannot be proven and we can merely view this as a surrogate for what might be occuring in other organ systems. It is also not possible to imply causation of the failure to achieve growth potential by reduced 5mC in observational studies such as mine. IGF2 is regarded a key *fetal* growth factor and its functional relevance in infancy is not well known however, recent data suggests there is indeed a role for circulating IGF2 levels (probably from both hepatic and non-hepatic sources) in postnatal growth (Begermann et al., 2015).

Certain genomic regions that acquire methylation marks during development are probably more susceptible to early life events than others. Exemplified here was the marked reduction in 5mC at *IGF2 DMR2* following preterm birth. It is a secondary imprinted region established post-fertilisatiion whereas imprinting marks at primary regions (such as *H19 ICR*) established during gametogenesis are generally maintained (Edwards & Ferguson-Smith, 2007). This study also added to evidence that stressors associated with lower socio-economic status can impact DNA methylation in early life (Borghol et al., 2012), here at *IGF2 DMR2*. This region was shown to be susceptible to nutritional modifications in a mouse study (Waterland,

2006) and overall it points towards the neonatal period being a 'programming window'.

The optimal growth pattern for preterm infants which would be associated with a favourable metabolic profile along with intact neurodevelopment is not known. A trial of a concentrated parenteral nutrition regimen with more glucose, protein and lipid (Standardised, Concentrated with Added Macronutrients Parenteral nutrition (SCAMP trial ISRCTN76597892)) promoted head growth in the neonatal period as the primary outcome in very preterm infants. And measures of 2 year neurodevelopmental outcomes in this study is awaited (Morgan et al., 2014). But paradoxically, preterm infants who were breastfed without supplementation in-hospital and post-discharge, had better head growth and neurodevelopmental outcome despite an initial drop in weight standard deviation score (Roze et al., 2012). The mechanism for these processes needs to be resolved for what might be seen as competing outcomes. In recognition of this, we explored but did not find a relation between 5mC at *IGF2/H19* and MR derived measures of white matter integrity or whole brain volume. The basis was that genomic imprinting has been implicated in neuropsychiatric disorders (Badcock & Crespi, 2008) which are regarded largely as diseases of white matter (Davis et al., 2003). Nevertheless, the pathway that was established for obtaining advanced magnetic resonance images of the preterm brain is now suitable for other mechanistic as well as therapeutic studies.

7.3 Telomeres

We investigated whether telomere attrition is an alternative mechanism for how early life stress 'gets under the skin' (Epel et al., 2004) and becomes a marker of increased disease risk. The were no differences in relative telomere length between the two groups by 1 year, and this was not suggestive of accelerated cellular ageing as a result of preterm birth, at least up until this time point. The full term infants demonstrated telomere lengthening over the first year, a phenomenon observed in other longitudinal studies (Shalev et al., 2012) (Epel et al., 2009), and this may be due to favourable lifestyle factors that up-regulate telomerase (Ornish et al., 2008). This is key considering the outpouring of work on telomeres in childhood and

adulthood (particularly with strong links to cardiovascular risk (Haycock et al., 2014)) and the paucity of data regarding early life. Telomere attrition is reported to be the fastest in the first 4 years of age (Frenck et al., 1998) (Zeichner et al., 1999), hence a time point thereafter may reveal larger discrepancies between the groups if preterm birth alters the trajectory of telomere attrition in the long term. Choice of assay and tissue for measuring telomere length in this context has been reviewed (Turner et al., 2014) and according to this, our choice of buccal epithelial cells and the qPCR method are acceptable. Modifications to this method have been developed to enhance accuracy, reduce throughput and to quantify absolute telomere length enabling comparison between laboratories (Cawthon, 2009) (O'Callaghan & Fenech, 2011) and should be considered in future.

7.4 DNA methylation and the placenta

Normal placentation is disrupted with preterm birth and this may be a reason for programmed effects – for example the lack of the placental barrier to glucocorticoids and the carefully controlled delivery of nutrients and growth factors. There has been a wealth of research studying the relationship between 5mC in the placenta and size at birth, but few studies have included birth weights across the full range. Several have included the measurement of both DNA methylation and gene expression, but none have investigated 5-hydroxymethylcytosine (5hmC) which has received much recent attention. The role of 5hmC in neurons and embryonic stem cells has been well studied (Mellén et al., 2012), however its role in the placenta is not known. As gestational age influences the methylome and gene expression (Novakovic et al., 2011) (Kumar et al., 2012), it was necessary to restrict to full term placental samples. Opposite patterns of gene expression in relation to birth weight were demonstrated for *IGF2* and *CDKN1C*, two oppositely imprinted genes in keeping with the 'parental conflict' hypothesis (Moore & Haig, 1991). Analysis of 5mC and 5hmC at the relevant regulatory regions showed associations with birth weight. Furthermore, the presence of 5hmC at loci commonly studied in the context of developmental programming in the placenta was confirmed. This was aided by the use of new techniques, chemical capture and immunoprecipitation, to estimate 5mC and 5hmC respectively and they discriminate between modifications that previous research

employing methods such as bisulphite conversion would not have been able to do (Huang et al., 2010).

7.5 Biomarkers of risk

Obtaining a biomarker of disease risk prior to the development of the phenotype would help the development and targeting of appropriate therapies. As 5mC at *IGF2/H19* in buccal epithelia does not appear to be stable, it may not serve as a biomarker, however other genomic regions may have more utility, as might a methylome wide 'signature' or a composite measure of phenotype and epigenotype. Similarly, a static measure of telomere length may not perform as well as a measure over time showing either attrition or lengthening. The placenta or umbilical cord blood, of course, can only serve as static measures. They would be more suited to full term neonates, as aspects of the neonatal course (e.g. late onset infection) have a bearing on outcomes for preterm infants, so samples at term corrected age or later are more useful. It may be necessary for diagnostic purposes to identify tissue specific profiles for diseases in general, but in infancy and childhood, buccal epithelial cells seem to be the most appropriate.

Very preterm infants are at the highest risk of long term morbidity. However late preterm infants are still at risk and although affected individuals are rare, late preterm infants are greater in number. These infants are not routinely followed up in medical clinics and along with very preterm infants, would greatly benefit from "precision medicine" by identifying biomarkers of risk early in life. This is more likely to be in the form of "precision prevention" with institution of educational support but also social support given the lower quality of life that these individuals endure into adulthood following on from social difficulties earlier in life (Baumann et al., 2016). Continued efforts should also focus on modifying behaviour such as encouraging physical activity and improving diet as individuals born preterm exercise less than their term born counterparts (Kajantie et al., 2010) despite ability to achieve normal exercise capacity in response to training (Clemm et al., 2012).

7.6 Intergenerational effects

The transmission of an environmentally acquired phenotype to subsequent generations has been well described in animal studies and one mechanism may be heritable epigenetic changes (Radford et al., 2014). Suggestions of intergenerational effects have also been reported in humans following prenatal exposure to the Dutch famine (Painter et al., 2008). Data is emerging showing that the full term children of adults born preterm, like their parents, have increased central adiposity (after adjusting for parental BMI) (Mathai et al., 2013), abnormal ambulatory BP (Mathai et al., 2015) but not insulin resistance (Mathai et al., 2012). The association would be further compounded should the offspring be born preterm, for which there is a recognised family risk (Bhattacharya et al., 2010) despite reduced reproductive capacity (Swamy et al., 2008). This is mostly via the maternal line, but the paternal line contributes as well (Wilcox et al., 2008). It is likely to be a genetic trait, but epigenetic modification may play a role. Future studies should consider obtaining paternal, in addition to maternal, biological samples to investigate intergenerational effects of preterm birth.

7.7 Future research

The current cohort of preterm and term infants and the biological samples gathered can be utilised to address the issues raised.

Whole blood samples (rather than serum) from this cohort at various time points in infancy (both cord and peripheral blood) kept in long-term storage could be utilised to determine whether altered DNA methylation at *IGF2* has a functional relevance by measuring circulating IGF2.

Additionally, genome wide analyses would yield other loci that are altered by preterm birth and information about whether any changes persist during infancy and beyond. Of interest would be pathways pertaining to inflammation given that this is common to both cardiovascular disease (Libby, 2006) and preterm brain injury (Volpe, 2009), and that very preterm birth has inflammatory antecedents (Goldenberg et al., 2000). Loci that retain altered DNA methylation in buccal cells should be studied in other tissues already collected - cord blood, peripheral blood and

placenta - to understand the tissue-specific nature of DNA methylation. Comparison with maternal blood collected can also be informative. Given the altered HPA axis activity in infancy, studying 5mC at regulatory regions of *NR3C1*, *HSD11β2* and *CRH* should be considered.

It would be important to conduct further follow up, ideally be at school age both before and after puberty. Cortisol secretion can be related to subtle differences in behavior and school performance that may be evident in the primary years. Thereafter it would be interesting to observe the trajectory of cortisol secretion in adolescence. Body composition should be assessed at both time points as children born preterm have been shown to demonstrate a greater increase in BMI than term born children during these years (Hack et al., 2011). In this regard, exercise and dietary intake should be measured. Follow up will allow the opportunity to study telomere length in relation to those in infancy; newer techniques can be trialed and telomerase activity should be measured. The extent to which the epigenome is fluid can be gleaned by studying DNA methylation over the life course including any changes that accompany the dramatic changes in development during puberty. It would then remain to be seen whether any changes in the epigenome associate with emerging risk factors for cardio-metabolic disease such as insulin resistance, hypertension and dyslipidaemia. Study over the life-course is required to establish whether there is an enduring legacy of preterm birth reflected in the epigenome.

Observational studies cannot establish causation but randomised controlled trials can asssist, particularly trials of nutritional interventions for this area of research. It may be informative to measure DNA methylation in participants in trials such as the SCAMP trial to investigate whether nutrition alters the methylome as it does for head growth. This may be an opportunity to identify genes or pathways that are implicated in both cardio-metabolic and neurological sequelae and point towards a common mechanism.

Developing and optimising MR techniques to quantify volume of brain regions such as the hippocampus and amygdala in early life and relating these measures to DNA methylation at *NR3C1* can be considered. Common genetic variants have been

identified that modify cognition, neuropsychiatric risk and white matter integrity (Caspi et al., 2007) (Kohannim et al., 2012) (Boardman et al., 2014). As genotype influences variation in DNA methylation (The et al., 2014), the study of gene-environment interactions may yield further clues about individual risk or resilience to disease.

With respect to the placenta, genome wide study to could identify other loci where hydroxymethylation may modulate fetal growth, but using newer techniques such as oxidative or TET assisted bisulphite sequencing that can offer single nucleotide resolution of hydroxymethylcytosine (Yu et al., 2012). Additionally, study of histone modifications or partially methylated domains have been under-explored. The study could be extended to compare the findings at full term with placentas from preterm deliveries that were collected at various gestational ages to better understand the role of DNA modifications during stages of human development.

7.8 References

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